

ISCHEMIA–REPERFUSION SYNDROME IN HUMAN RENAL TRANSPLANTATION

Studies of the Pathophysiological Mechanisms

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ACADEMIC DISSERTATION

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To my Family

A human being should be able to change a diaper, plan an invasion, butcher a hog, conn a ship, design a building, write a sonnet, balance accounts, build a wall, set a bone, comfort the dying, take orders, give orders, cooperate, act alone, solve equations, analyze a new problem, pitch manure, program a computer, cook a tasty meal, fight efficiently, die gallantly. Specialization is for insects.

- Robert A. Heinlein

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ABSTRACT

Renal transplantation represents the treatment of choice for the majority of patients with end-stage renal disease. Since the introduction of calcineurin inhibitor immunosuppressive drugs in the 1980s, we have witnessed a successful era for all solid organ transplantations. However, many kidneys continue to fail resulting from chronic allograft nephropathy years after transplantation. Increasing evidence from the last three decades suggests that early graft injury also jeopardizes long-term outcomes. As demand for renal transplants continues to rise, a better understanding of early allograft injury is required in order to develop protective interventions.

This thesis focuses on the inflammatory mechanisms of early kidney graft injury as well as the differences in systemic inflammatory responses in patients following various immunosuppressive protocols. In total, 45 adult patients undergoing renal transplantation at Helsinki University Hospital were studied. Patients were recruited as a subset from a larger randomized study comparing three immunosuppressive protocols. Thus, 15 (group A), 14 (group B), and 16 (group C) patients were consecutively recruited retaining the original randomization. In group A, patients received perioperative a 9-mg/kg infusion of antithymocyte globulin complemented by a triple immunosuppression therapy consisting of reduced dose cyclosporine, azathioprine, and steroids. Group B patients received two doses of the interleukin 2 (IL-2) receptor antagonist basiliximab with a reduced dose cyclosporine triple therapy as in group A. Group C patients received a conventional triple immunosuppression therapy of 10-mg/kg cyclosporine, azathioprine, and steroids.

Central vein blood samples were taken before surgery, before graft reperfusion, and during reperfusion. In addition, during reperfusion blood samples were also obtained directly from the renal graft artery and vein in order to study phenomena local to the graft vascular bed. From blood samples, various established biomarkers of inflammation and coagulation were determined complemented with measurements of the graft blood flow.

The primary findings from this study consisted of the associations between the investigated inflammatory markers and the pathways with ensuing delayed graft function (DGF). Marked neutrophil sequestration in the renal graft was demonstrated immediately after reinstitution of the blood flow. Later, this was associated with a diminishing blood flow. Antithymocyte globulin (ATG) administration provided an unforeseen but unique opportunity to observe graft reflow under a systemic pro-inflammatory state. In this situation, avid graft uptake of APC was noted. Thus, this uptake may prove protective. Therefore, as a pharmaceutical preparation already exists, albeit for a different indication, new therapeutic interventions in the context of transplantation could be explored in the future.

ABSTRACT IN FINNISH

Valtaosalle loppuvaiheeseen edennyttä munuaistautia sairastavista potilaista paras hoitovaihtoehto on munuaisensiirto. Kuusikymmentäluvun kokeellisesta hoitomuodosta on kehittynyt nykypäivään tultaessa vakiintunutta toimintaa. Syklosporiini-lääke otettiin käyttöön neljä vuosikymmentä sitten, minkä jälkeen olemme saaneet todistaa elinsiirtojen voittokulkua. Edelleen kuitenkin vaikea ongelma on siirtomunuaiseen kehittyvä krooninen hyljintä, jonka vuoksi vuosien mittaan merkittävä määrä siirteistä menetetään. Noin kolmen vuosikymmenen tutkimusaineisto osoittaa, että siirteen varhaiset vauriot altistavat siirteen toiminnan heikkenemiselle ja siirteen menetykselle myös pitkällä aikavälillä. Koska siirtoa odottavia potilaita on jatkuvasti enemmän kuin tarjolla olevia siirteitä, parempi varhaisvaiheen vaurioiden ymmärtäminen olisi tärkeää suojaavien hoitojen ja toimenpiteiden kehittämiseksi.

Tämä väitöskirjatutkimus keskittyy siirtomunuaista varhaisvaiheessa vaurioittavan tulehdusreaktion mekanismeihin, sekä eri hylkimisenestolääkityksiä käyttävien potilasryhmien tulehdusvasteiden eroihin. Tutkimukseemme osallistui neljäkymmentäviisi aikuista, joille tehtiin munaisensiirto Helsingin yliopistollisessa keskussairaalassa. Potilaat rekrytoitiin osana tutkimusta, jossa vertailtiin kolmea erilaista hylkimisenestolääkitysohjelmaa. Potilasrekrytointi suoritettiin peräkkäin kustakin hylkimisenestolääkityksen ryhmästä säilyttäen alkuperäinen ryhmien satunnaistaminen. Ryhmässä A (15 potilasta) käytettiin leikkauksenaikaista 9mg/kg ATG infuusiota, alkuvaiheen matalaa syklosporiini-annostusta, atsatiopriinia ja kortikosteroideja. Ryhmässä B (14 potilasta) potilaat saivat kaksi infuusiota IL-2 -reseptorin estäjää basiliximabia ja muuten samanlaista, alkuvaiheessa matala-annoksista syklosporiini-kolmoishoitoa kuten ryhmän A potilaatkin. Ryhmän C potilaat (16 potilasta) saivat traditionaalista kolmoishoitoa 10mg/kg syklosporiini-annostuksella, atsatiopriinilla ja kortikosteroideilla.

Verinäytteet otettiin keskuslaskimosta ennen leikkausta, ennen siirteen reperfuusiota ja reperfuusion aikana. Tämän lisäksi reperfuusion aikana otettiin verinäytteet siirteen valtimosta ja laskimosta siirteen paikallisten ilmiöiden tarkastelemiseksi. Verinäytteistä määritettiin verenkuvaa sekä neutrofiilien ja monosyyttien CD11b ja L-selektiini –ekspressiot virtaussytometrialla. Edelleen plasmasta määritettiin laktoferriini-, tPA-, D-dimeeri-, protrombiinifragmentti 1 + 2 (F1+2), PAI-1, MMP9- ja TIMP-1 –pitoisuudet entsyymi-immunologisesti (ELISA). Proteiini-C ja aktivoitu proteiini-C määritettiin entsyymi-kaappaus –metodilla. Verenvirtaus siirteessä mitattiin pulssi-doppler virtausmittarilla.

Tutkimuksen keskeisimmät tulokset koskevat tutkittujen markkerien assosiaatioita viivästyneeseen siirteen käynnistymiseen. Välittömästi verenkierron aukaisuun liittyen todettiin huomattava neutrofiilisolujen kertyminen siirteeseen. Tähän puolestaan liittyi

viiveellä siirteen verenkierron heikentyminen. ATG-annostelu aiheutti ennalta-arvaamattoman proinflammatorisen reaktion, mikä tarjosi kiinnostavan mahdollisuuden tutkia munuaissiirteen verenkierron käynnistymistä systeemisen tulehduksen vallitessa. Tutkimuksessamme totesimme ATG-ryhmässä siirteen kuluttavan runsaasti kiertävää aktivoitunutta proteiini-C:tä. On mahdollista, että aktivoituneen proteiini-C:n vaikutus voi tässä tilanteessa olla suojaava, mikä tarjoaa mahdollisuuden tulevaisuudessa lääkehoitotutkimuksiin.

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LIST OF ABBREVIATIONS

ADH	Antidiuretic hormone
AIM2	Absent in melanoma 2
APC	Activated protein C
ATG	Antithymocyte globulin
ATP	Adenosine triphosphate
BPI	Bactericidal permeability increasing protein
CLR	C-type lectin receptor
CsA	Cyclosporin A
DAMP	Damage-associated molecular pattern
DCD	Donation after cardiac death
DGF	Delayed graft function
ECD	Expanded criteria donor / donation
ELISA	Enzyme-linked immunosorbent assay
EPCR	Endothelial protein C receptor
FITC	Fluorescein isothiocyanate
F1+2	Prothrombin fragment 1 + 2
HIF	Hypoxia-inducible factor
HLA	Human leukocyte antigen
HMGB1	High-mobility group box 1
HUH	Helsinki University Hospital
HUSLAB	Helsinki University Laboratories
ICAM-1	Intercellular adhesion molecule 1
IL-2	Interleukin
I/R	Ischemia/reperfusion injury
LPS	Lipopolysaccharide
LRD	Living related donation
MAC	Membrane attack complex
MMP	Matrix metalloproteinase
NF- κ B	Nuclear factor kappa B
NOD	Nucleotide-binding oligomerization domain
NLR	NOD-like receptors
PAI-1	Plasminogen activator inhibitor 1
PAMP	Pathogen-associated molecular pattern
PAR-1	Protease-activated receptor 1
PC	Protein C
PE	Phycoerythrin
PHD	Prolyl hydroxylase

PRA	Panel reactive antibodies
PRR	Pattern recognition receptor
RAGE	Receptor for advanced glycation end products
RFU	Relative fluorescence unit
RIG-1	Retinoic acid inducible gene 1
RLR	RIG-1-like receptor
ROC	Receiver operator characteristic
ROS	Reactive oxygen species
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TIMP-1	Tissue inhibitor of matrix metalloproteinase 1
TLR	Toll-like receptor
TNF	Tumor necrosis factor
tPA	Tissue plasminogen activator
UNOS	United Network of Organ Sharing

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

- I. Association of graft neutrophil sequestration with delayed graft function in clinical renal transplantation. Turunen AJ, Lindgren L, Salmela KT, Kyllönen LE, Mäkisalo H, Siitonen SM, Pesonen EJ. *Transplantation*. 2004 Jun; 77(12):1821–6.
- II. Activated protein C reduces graft neutrophil activation in clinical renal transplantation. Turunen AJ, Fernández JA, Lindgren L, Salmela KT, Kyllönen LE, Mäkisalo H, Griffin JH, Siitonen SM, Petäjä J, Pesonen EJ. *Am J Transplant*. 2005 Sep; 5(9):2204–12.
- III. Intragraft coagulation events and delayed graft function in clinical renal transplantation. Turunen AJ, Lindgren L, Salmela KT, Kyllönen LE, Petäjä J, Pesonen EJ. *Transplantation*. 2008 Mar; 85(5):693–9.
- IV. Matrix metalloproteinase 9 and graft preservation injury in clinical renal transplantation. Turunen AJ, Lindgren L, Salmela KT, Kyllönen LE, Andersson S, Pesonen E. *Transplantation Proc*. 2015 Dec; 47(10):2831–5.

The publications are referred to in the text by their Roman numerals.

INTRODUCTION

Since the clinical introduction of calcineurin inhibitors in 1983, solid organ transplantation has become a highly successful treatment modality for various organ failure states. During this “calcineurin inhibitor era”, a dramatic increase in our understanding of human immunology accompanied by an increase in the absolute number of clinical transplantations performed worldwide occurred. Simultaneously, it has become painfully apparent that a further rapid increase in transplant demand has also occurred. In part, this is due to the rising incidence for diseases such as hepatitis C and type 2 diabetes, frequently leading to organ failure. Moreover, as surgical techniques, immunosuppressive regimens, and intensive care treatment methods improved, indications for organ transplantation have broadened. In developed countries, the human life span is constantly lengthening and increasingly older patients with sometimes complex comorbidities are now considered eligible for organ transplantation.

Thus, it is evident that while advanced immunosuppressive regimens are used, many grafts continue to fail in the long term and individual patients may need multiple sequential transplants (*Friedersdorff et al. 2016*). Increasing pressure falls on transplant service—providing organizations struggling with waiting lists. Disappointingly, thus far chronic graft failure research remains quite insufficient and protective interventions are lacking.

In an effort to counter the urgent need for more organs, we have now seen a stepwise extension of donor criteria. Among the more recent techniques employed, transplant programs now rely not only on brain-dead donors but also donors following cardiac death. Compared to more conventional organ donation, grafts from these new donor groups are likely to be further stressed or injured prior to organ procurement. Accordingly, these grafts may experience more problems vis-à-vis initial function and may be at higher risk for failure in the long term. Yet, with careful recipient selection, even these unconventionally sourced transplants result in survival benefits for recipients.

Since the early 1990s, the theoretical understanding of immunology has shifted from strict notions of self–non-self discrimination. It is now understood that mammalian immune system responses depend not only on recognition of foreign elements but also detection of “danger” signals encountered simultaneously. Necrotic cell death is one example of a danger signal. (*Matzinger 1994*). When considering the entire organ transplantation process, this shift highlights the pathological processes that take place early on while still in the operating theater. The early phases of the procedure are not only crucial to the initial graft function, but may also comprise an injurious primary event setting up a smoldering host response,

potentially culminating in repeated episodes of acute rejection and chronic graft failure much later. This view is supported by long-standing epidemiological evidence.

In Finland, a clear majority of renal grafts originate from cadaveric donors and, thus, undergo various lengths of ischemic preservation followed by reperfusion. The resulting ischemia–reperfusion (I/R) injury is exceedingly complicated and multifactorial. Despite intensive research, it also remains poorly understood. In general, organ preservation and I/R injury appear to activate cellular elements of innate immunity, soluble components of inflammation and coagulation, and paracrine mediators of epithelial, endothelial, and extracellular matrix homeostasis. Taken together, these early activated mechanisms work towards re-establishing intra-organ homeostasis, while simultaneously carrying the potential for considerable further local tissue damage and priming for subsequent attacks from the adaptive immunity arm.

This study was conducted in a typical clinical transplantation practice setting in order to further elucidate select cellular and sub-cellular mechanisms leading to renal I/R injury.

REVIEW OF LITERATURE

CLINICAL ASPECTS

History

In Finland, the first renal transplantation was performed in 1964. A mere year earlier, the Belgian surgeon Alexandre for the first time recovered and transplanted a kidney from a heart-beating brain-dead donor, well before any legislation regarding brain death was adopted (*Machado 2005*). Since then, more than 6000 renal transplantations have taken place in Finland, while annually 180 to 260 transplantations are performed.

In 1971, Finland became the first country to adopt the concept of brain death both medically and legally. To date, more than 95% of all renal grafts originate from deceased brain-dead donors, exceeding 130 annually for the first time in 2016. However, during the last 25-year period, the number of recipients on the transplant waiting list has doubled, and exceeded 400 at the time of writing (*Scandiatransplant statistics 2013–2016*). Waiting list times have similarly increased, with the median time spent waiting exceeding one year for the primary transplant. In the United States, the corresponding waiting time is at least threefold higher (2011 UNOS Annual Report). Over the past four years in Finland, a maximum of 11 patients each year died while waiting for a renal transplantation. An amendment to the Finnish law on organ transplantation, now assuming presumed consent for organ donation, was passed in 2010. While the long-term effect of the amendment on the number of deceased donors awaits evaluation, the most recent annual figures appear to indicate a fair increase in the number of donor organs. Traditionally, donations from a spouse or living relative have not been popular and were usually considered only for pediatric recipients. Very recently, some momentum has been gained in this respect, however.

Indications

Absolute contraindications for renal transplantation are few. Each individual end-stage renal disease patient should be evaluated for transplantation, preferably during the pre-dialysis phase of disease (*European Best Practice Guidelines Expert Group on Renal Transplantation 2000*). For the patient, transplantation offers the best renal disease-related prognosis as well as the best quality of life (*Wolfe et al. 1999, Sarnak et al. 2003*). The cost-effectiveness of transplantation is excellent, whereby expenditures associated with hemodialysis typically exceed transplantation-related costs in six months (*Salonen et al. 2003*). In Finland, almost all adult renal transplantation candidates already depend on maintenance dialysis once placed

on the transplant waiting list. Among pediatric candidates, a pre-emptive transplantation is sometimes performed without proceeding to dialysis. Table 1 summarizes the diagnoses related to terminal uremia for adult patients in Finland.

Table 1.

Distribution of diagnoses (%) related to end-stage renal disease leading to transplantation among adults in Finland during 2000–2009 and 2010–2016.

Diagnostic group	2000-2009	2010-2016
Glomerulonephritis	23	22
Polycystic degeneration	18	17
Type 1 diabetes	21	19
Type 2 diabetes	5	7
Tubulointerstitial nephritis	6	3
Amyloidosis	2	1
Other	20	22
Undefined	6	8
Total	100	100
N	1772	1491

Table courtesy of Associate professor Patrik Finne, MD PhD, director of the Finnish liver and kidney association.

Organ donation

In Finland, most kidney allografts originate from deceased brain-dead donors, while multi-organ procurement techniques are routinely utilized. A considerable number of the grafts to date already result from the so-called expanded criteria donors (ECDs) as defined by the United Network for Organ Sharing (UNOS) (*Metzger et al. 2003*). Thus far, donors after cardiac death (DCD) or other non-heart-beating donors have not been used. Organs are allocated based on ABO blood group matching and human leukocyte antigen (HLA) tissue types, white blood cell cross match, and time spent on the waiting list. Finnish transplantation candidates enjoy benefits stemming from the relatively straightforward co-operation between the single transplantation center at Helsinki University Hospital and referring hospitals as well as the relatively short geographic distances between facilities. Both facts contribute to shorter preservation times. HLA matching is exclusively accomplished at a single center and, thus, does not result in excessive organ preservation time given that tissue types are typically already known prior to organ procurement surgery.

The ideal allograft kidney donor is a young, living relative with a good HLA match. However, in Finland, living related donation (LRD) has not gained in popularity and a vast majority of adult renal grafts originate from deceased brain-dead donors. This contrasts somewhat to trends

elsewhere in Europe and particularly in the United States, where the number of living kidney donors surpassed the number of deceased donors in 2000 (*2011 UNOS Annual Report*).

In comparison, the ideal deceased donor then is a younger person, who dies from a traumatic head injury isolated to the brain, leaving the thoracic and abdominal organs intact. In Finland, transplantation clinicians have recently raised concerns on the probable suboptimal use of potential donors (*Salmela 2010*). As such, even the most active donor-referring hospitals in Finland assessed by an internationally verified self-assessment program (*Roels & Wight 2001*) have found their performance to be less than optimal. Reflecting this, the best donor-referring hospitals continually recognize and refer more than twice the number of donors per million population compared with the least-active referring units (*Isoniemi H, lecture commentary, national transplantation convention, Helsinki 2012, Höckerstedt et al. 2005*). Some estimates suggest that the number of Finnish patients on organ transplant waiting lists could be substantially reduced if all donor-referring hospitals performed optimally in terms of recognizing potential donors. Many reasons can be attributed to the suspected suboptimal recognition of donors. For instance, ever-increasing pressure towards better cost-efficiency in healthcare may leave potential donors overlooked, since deceased patients must be admitted to intensive care units until organ procurement occurs.

By comparison, taking Norway's example, a Nordic country much like Finland including its population size, improving the Finnish deceased donor identification appears possible. During the four-year period between 2010 and 2014 in Norway, 22 to 26 deceased donors per million inhabitants were annually recruited as opposed to the 17 to 22 per million inhabitants in Finland (*Scandiatransplant statistics 2011–2016*). This situation has been reversed in the last two years, however, as Finland identified 22 to 24 donors per million inhabitants compared with the Norwegian figures of 20 to 22 per million inhabitants. Complemented by an exceptionally active Norwegian program for living donation, significantly more kidneys have been historically transplanted in Norway; yet, in 2016, a higher total number of transplantations took place in Finland. The annual number of Norwegian patients who died while wait-listed compares with figures from Finland vis-à-vis the total number of patients wait listed for renal transplantation. Finland cooperates in Scandiatransplant, which is aimed at organ sharing across the Nordic countries (*Grunnet et al. 2001*). Yet, in practice, only about ten Finnish kidneys are exchanged annually through this collaboration (*Salmela et al. 2004*). All Nordic countries maintain their own national waiting lists.

Since at least 2002, the so-called expanded criteria donor (ECD) for kidney donation has been relatively well described (*Metzger et al. 2003*). A considerable number of all kidneys donated in Finland already originate from ECD-derived sources. Compared with maintenance dialysis, the long-term feasibility of such transplantations has been documented (*Ojo et al. 2001*), although the overall relative risk of graft failure exceeds 1.7 compared with standard donor

kidneys. However, decision-making related to ECD kidney transplantation for individual cases remains unambiguous. For instance, relative mortality risk analysis shows that the short-term risk of death following transplantation is more than five times greater when compared to standard therapy with dialysis while waiting for a non-ECD kidney. This risk reverses only after 226 days following transplantation and, thus, cumulative mortality does not equalize until 3.5 years after ECD transplantation (*Merion et al. 2005*). Beyond this period, however, the ECD-transplanted patient population begins to exhibit an overall survival benefit. In North America, these prognostic disparities led to policies calling for patient informed consent before becoming eligible for an ECD kidney (*Metzger et al. 2003, Pomfret et al. 2008*). Similar concerns were also raised in Europe (*Bruzzzone & Venettoni 2008*). However, in the United States, a new policy utilizing the expedited allocation of ECD kidneys proved effective in addressing an organ shortage given the considerable rise in donations observed (*Sung et al. 2005*). Furthermore, experiences from densely populated regions of Europe suggest that decision-making without strict criteria can be misleading and, consequently, viable grafts may be discarded (*Friedersdorff et al. 2013*).

Due to the tremendous shortage of organs, even more marginal donors are currently utilized internationally. These include DCD donors and donors that die after brain death but before formal organ procurement procedures have been initiated (*Rao & Ojo 2009*). In select cases, transplantation using these presumably more compromised organs can be feasible, particularly when compared to patients still dependent upon maintenance dialysis (*Summers et al. 2015*). Concerns, however, regarding the appropriate use of such organs have been voiced, given that organ demand rather than careful planning before plantation tend to drive clinical practices (*McDonald & Clayton 2013*). The immediate costs of transplantation services increase when such marginal donors are utilized, although for kidney transplantation savings over maintenance dialysis remain relevant (*Pomfret et al. 2008*). In Finland, DCD donors have not been utilized thus far and the Finnish transplantation community maintains that the most expedient means of expanding the donation pool should focus on a concerted effort to utilize traditional donors more effectively and encouraging LRD in carefully considered and select cases (*Salmela 2010*). By contrast, Norway has adopted an alternate policy, becoming the first Nordic country to initiate DCD transplantations in 2015.

Utilizing DCD donors inevitably introduces a significant warm ischemic component to the renal graft ischemia–reperfusion (I/R) injury. Experimentally, it is clear that increasing warm ischemia associates with more frequent primary non-functioning and delayed graft function (DGF) problems. However, in a relevant porcine model, even a 120-min warm ischemic insult did not translate into histological necrosis (*Hosgood et al. 2015*). Renal function deteriorated severely after just 60 min of warm ischemia, though. In any case, warm ischemia resistance of the human kidney could be better than previously expected (*Parekh et al. 2013*), and, thus, the hope of relying increasingly on DCD donor sources might be warranted.

Surgery

In Finland, organ procurement surgery is performed exclusively by the surgical teams of the only transplantation center in the country. These teams travel to donor-referring hospital locations. Standard multi-organ procurement techniques are utilized, although perfused preservation techniques are not.

The recipient operation is performed as described by Salmela (1994) preferably with end-to-side vascular anastomoses to the recipient's external iliac artery and vein. Traditionally, open ureteroneocystostomy is performed. Rarely, perhaps once a year, a situation emerges whereby renal transplantation is attempted, but, due to extreme recipient atherosclerosis, no renal graft can be implanted (*Salmela, personal communication*).

Immunosuppression

Modern immunosuppressive agents—primarily mycophenolate and tacrolimus—are currently used in addition to more traditional immunosuppression by cyclosporine, azathioprine, and corticosteroids. In the United States, horse-derived antithymocyte globulin (ATG) is often used as an adjunct to the initial immunosuppressive regimen (*Hardinger 2006*). In Europe, a similar product, rabbit-derived ATG, is available and used in select protocols with beneficial effects (*Kaden et al. 2009*). In this thesis, participating patients were recruited from a larger study, where two immunosuppressive agents not traditionally used in Finnish immunosuppressive induction were utilized and studied. These agents consisted of ATG (Fresenius) and the interleukin-2 (IL-2) receptor blocking antibody basiliximab (*Kyllönen et al. 2007*).

Fresenius ATG, briefly, is a polyclonal antibody infusion created in rabbits immunized with Jurkat cells, an established line of immortalized T-lymphocytes originally obtained from the peripheral blood of a 14-year-old boy with leukemia. Different ATG preparations are each subject to research, since a multitude of clonal specificities are raised and the biological effects accordingly vary (*Popow et al. 2012*). Clinically, these preparations have been used in transplantation since the 1970s, whereby their main effects include not only T-lymphocyte depletion, but also non-depletive immunomodulation (*LaCorcia et al. 2009*). The adverse effects include cytokine release syndrome, which may require pre-treatment with corticosteroids (*Kaden 2002, Denny et al. 2015*). ATG preparations are more popular in the United States than in Europe, where interest in Germany in particular is more focused on developing new dosing algorithms. Of specific interest in this thesis, ATG preparations appear

to have an I/R injury protective efficacy beyond lymphocyte ablative effects. Namely, antibody specificities against selectin, integrin, and immunoglobulin superfamilies expressed in the endothelial cells and leukocytes have been identified (*Rebellato et al. 1994, Michallet et al. 2003*). Furthermore, in an experimental setting, diminished leukocyte vascular adhesion with a better sustainability of blood flow has been observed by intravital microscopy (*Chappell et al. 2006*).

Basiliximab is a recombinant murine/human chimeric nondepleting IgG1 monoclonal antibody directed against the IL-2 receptor. The IL-2 receptor was the first interleukin receptor characterized. It is present on the surface of activated T-cells, where upon IL-2 binding the receptor subunits converge to form a stable quaternary macromolecule with intracellular domains. Three intracellular signaling pathways are subsequently initiated. If the IL-2 stimulus is strong enough to reach a critical number of receptors triggered on the cell surface, T-cell DNA replication and mitosis occur (*Malek & Castro 2010*). Pharmacological intervention can be delivered in the form of basiliximab, which interrupts the abovementioned signal transduction chain and, thus, prevents the clonal expansion of alloreactive T-cells (*McKeage & McCormack 2010*).

Delayed graft function (DGF)

It is not uncommon for a transplant surgeon to observe immediate graft function. For instance, urine output may be observed already on the operating table, at times even from the sectioned end of the graft ureter awaiting suturing to the bladder. Unfortunately, the contrary is also common: Halloran *et al.* (1988) defined delayed graft function (DGF) as a plasma creatinine concentration $\geq 500\text{mmol/l}$ throughout the first week post-transplant, requiring more than one dialysis session during the first week post-transplant, or oliguria $\leq 1\text{ l/24 h}$ for more than two days. Traditionally, this phenomenon has been viewed as the clinical manifestation of I/R injury. Furthermore, concrete evidence links DGF to inferior long-term outcomes (*Ojo et al. 1997, Gill et al. 2016*). Some researchers suggest that DGF associates with the same reduction in graft survival and graft half-life as a full HLA mismatch (*Halloran & Hunsicker 2001*). Accordingly, focusing on decreasing the cold ischemia time by accepting less optimal tissue matching may translate into more kidneys being transplanted (*Port et al. 2002*). In a study of more than 6000 renal transplantations, no significant tissue matching gains were identified when grafts travelled longer distances, but the associated longer cold ischemia time translated into worse long-term outcomes (*Salahudeen et al. 2004*). It appears, however, that HLA mismatching in itself may actually constitute a risk factor for DGF (*Kamoun et al. 2008*), thus highlighting the complex pathophysiology behind this clinical diagnosis.

In Finland, gradual improvements mitigating the incidence of DGF have been observed in recent decades. In the 1980s, nearly 40% of renal grafts suffered from DGF. However, procurement techniques evolved and related surgeries were performed almost increasingly and exclusively by dedicated transplant surgeons. This, in part, contributed to DGF incidence holding steady at 25%, despite the almost exclusive use of deceased donors (*Salmela & Kyllönen 2007*). However, because the use of ECD kidneys has become more commonplace, incidence is again rising. In a study from our institution comprising 176 consecutive deceased donor kidney transplantations, the observed incidence of DGF reached 40% (*Hollmen et al. 2011*). As expected, DGF also translated into worse one-year graft survival at 90.0% vs. 99.1% in this particular patient cohort.

Outcome

Renal transplantations account for 80% of all solid-organ transplantations in Finland. Outcomes for primary renal transplantations are excellent, whereby 95% of kidneys function properly one year post-transplantation and 87% function properly five years following transplantation, rates comparable with international figures (*Salmela et al. 2004*). In Figure 1, improving prognosis of renal transplantation by five year periods over time is displayed.

Figure 1.

Trends for renal graft prognosis during the cyclosporine era in Finland, 1985 to 2009, n=3749.

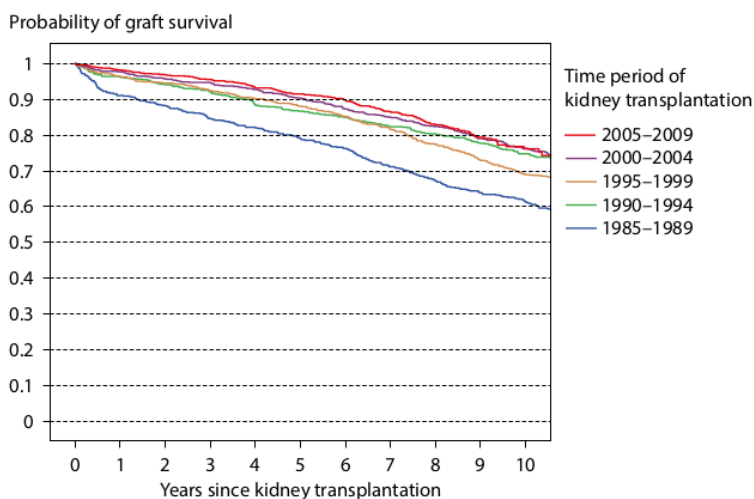
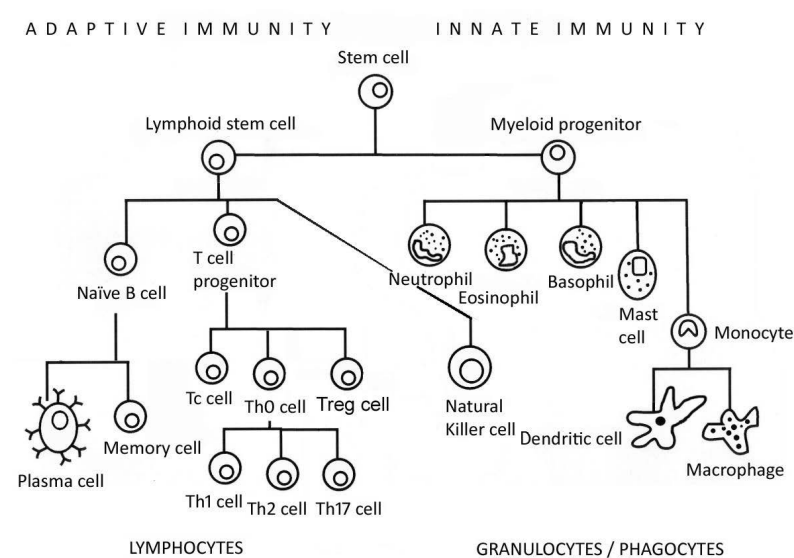


Table courtesy of Associate Professor Patrik Finne.

INNATE IMMUNITY

The immune systems in jawed vertebrates are divided into the innate and adaptive arms (Figure 2). The complement system and phagocytic white cells comprise the innate arm, while lymphocytes in concert with their antibody production comprise the adaptive arm. Based on the contemporary research, however, this division is overtly simplified as the processes of antigen presentation, effector function selection, tolerance induction, and the resolution of inflammation inarguably involve both arms (*Matzinger 1994, Nathan 2006*). However, the relevance of this division can be appreciated in the types of responses to repeated stimuli. Innate immune responses are similar when repeatedly encountering the same stimulus. By contrast, adaptive immune responses evolve and enhance upon repeated stimulation. However, as adaptive immune responses are initiated by innate immune reactions, the innate immunity responses triggered within the context of allogeneic organ transplantation are especially important.

Figure 2. Simplified overview of the development of immune system cells.



Adapted from “Systemic inflammation in preterm infants”, Riikka Turunen, academic dissertation ISBN 978-952-92-5499-6 (pbk.) ISBN 978-952-10-5499-0 (PDF). Helsinki University Printing House, Helsinki 2009. Reprinted with permission from the copyright holder.

Phagocytic white cells form the essential cellular elements of defensive innate immunity directed towards microbial infections or non-infectious tissue injuries. Phagocytes include monocytes/macrophages and neutrophilic polymorphonuclear white cells (that is, neutrophils). Eosinophils, basophils, and mast cells are traditionally considered a part of innate immunity, but their roles remain more obscure and are best described in relation to allergic and parasitic conditions.

The cells of innate immunity respond to and are recruited by tissue through various distress signals that act in a paracrine manner. Distressed parenchymal and endothelial cells can secrete and display cytokines or other chemotactic molecules as a call for help. In addition to being actively secreted by the parenchymal distressed cells, activating signals may also be detected as conserved structural moieties found in microorganisms. Collectively, these structures are then called pathogen-associated molecular patterns (PAMPs). One such PAMP is lipopolysaccharide (LPS), a long-known potent biomolecule behind clinically observed dramatic symptoms of sepsis. In addition, host-derived non-infectious material can trigger innate immunity responses in a similar manner. In this case, molecular patterns are called damage-associated molecular patterns (DAMPs), often involving structures normally situated in the intracellular compartment and nucleus (*Bianchi 2007, Lotze et al. 2007*). Due to uncontrolled cell death or injury, these structural elements are released into the extracellular space and, thus, become visible to elements of innate immunity (*Chen & Nuñez 2010*).

Innate immunity cells recognize PAMPs and DAMPs via an extensive array of conserved germline encoded receptors. Collectively, these receptors are called pattern recognition receptors (PRRs). To date, five classes of PRRs have been identified: toll-like receptors (TLRs) (*Leifer & Medvedev 2017*), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) (*Claes et al. 2015*), retinoic acid inducible gene I (RIG-I)-like receptors (RLRs) (*Weber 2015*), C-type lectin receptors (CLRs) (*Mayer et al. 2017*), and absent in melanoma 2 (AIM2)-like receptors (*Burckstummer et al. 2009*). PRRs have been intensely investigated since the landmark discovery of the toll gene's importance in *Drosophila* fungal infection resistance in 1996 (*Lemaitre et al. 1996*). In most cases, TLR activation causes dimerization of TLRs through their ligands, leading to intracellular signal transduction (*De Nardo 2015*). Today, it is understood that different PRRs can function together to form various heterodimers or participate in the formation of highly complex intracytosolic signaling aggregates with other proteins to form so-called inflammasomes (*Latz et al. 2013*). The formation of higher-order complexes, in part, serves to diversify the array of ligands that PRRs can recognize. PRR-triggered signal transduction pathways exhibit complex downstream regulation at the cytosolic and transcriptional levels. Broadly, the recognition of external and intrinsic danger signals by PRRs culminates in the activation of specific transcription factors or proteolytic pathways, resulting in the production of pro-inflammatory mediators such as the tumor necrosis factor (TNF) and IL-1.

Of special interest within the context of transplantation is the initiation and propagation of sterile inflammation. In solid organ transplantation, the most obvious injurious element is organ preservation–related ischemia and subsequent I/R injury. Prototypical DAMPs resulting from cells losing their membrane integrity include the chromatin-associated protein high-mobility group box 1 (HMGB1) (*Lau et al. 2014*), S100 calcium-binding proteins (*Foell et al. 2007*), heat shock proteins, purine metabolites, and uric acid. To further complement the recognition of these endogenous elements by PRRs, specific receptors for DAMPs also exist. One such receptor is the multiligand receptor for advanced glycation end products (RAGE), which recognizes products accumulating under high-oxidant stress conditions (*Chen & Nuñez 2010, Dessing et al. 2012*). In the context of renal I/R injury, TLR-4 has increasingly been shown to play an important role (*Kruger et al. 2009, Zhao et al. 2014*).

Neutrophils

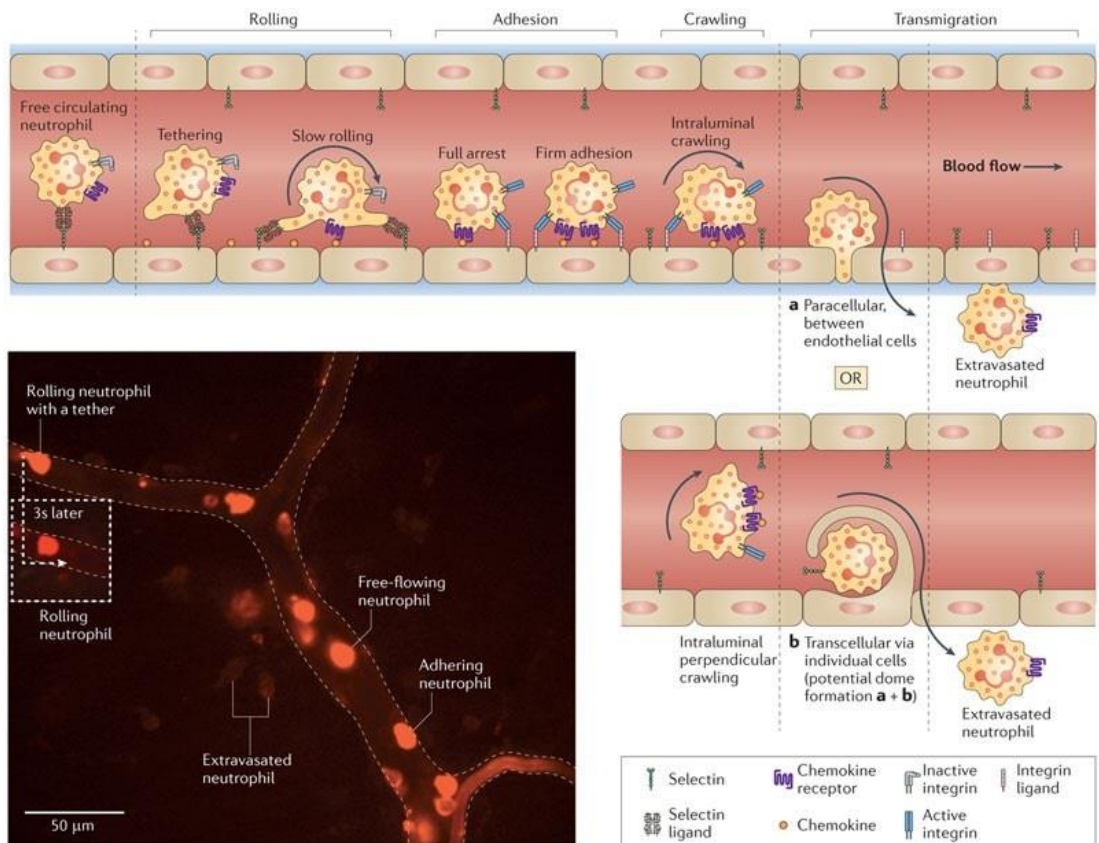
Neutrophils represent the first-line of defense against invading microbes. Their most natural adversaries consists of hostile bacteria, which neutrophils recognize aided by PRRs. Neutrophils are the most numerous leukocytes in the blood, although they are not typically present in healthy tissues and, as such, serve as the hallmark of acute inflammation in histological sections.

The organelle apparatus of a mature neutrophil appears not to carry extensive synthetic capabilities since the Golgi apparatus is small, ribosomes and mitochondria remain sparse, and the rough endoplasmic reticulum is altogether absent. However, some unforeseen capabilities, including cytokine production, are now understood to occur (*Nauseef 2016*). In large part, the actions of neutrophils rely on the timely expenditure of preformed granules, whereby each neutrophil contains roughly 200 granules (*Cowland & Borregaard 2016*). These organelles fall within the categories of primary, secondary, and tertiary granules, as well as secretory vesicles. More simplistically, the primary granules contain myeloperoxidase, bactericidal permeability increasing protein (BPI), defensin, elastase, and cathepsin G. Secondary granules contain lactoferrin and lysozyme. Finally, tertiary granules contain cathepsin and gelatinase B (also known as matrix metalloproteinase 9, MMP-9). Secretory vesicles, then, represent a quickly available reservoir of adhesion molecules, since the molecules contained are already membrane-bound so that their adhesive domains point towards the vesicle center. Vesicle fusion with the outer-cell membrane then reverses molecule orientation. Neutrophils are relatively short-lived cells with a life span estimated at between 24 h and 5.4 days. The make of neutrophils reflect their principal mission—they are armed for a one-way microbicidal mission.

Typically, when a blood-borne neutrophil encounters activated endothelium with increased adhesion molecules and chemokines, it establishes a cascade of adhesive interactions with endothelial cells lining the lumen of the postcapillary venular walls, ultimately exiting the blood vessel (*Ley et al. 2007, Muller 2016*). Initially, neutrophil is captured and weak selectin-mediated adhesions form allowing it to roll over the endothelial surface. Rolling slows the cell down, thus permitting surveillance for more chemotactic or other activating signals. If the stimuli encountered are sufficiently strong, neutrophil becomes primed, thereby allowing for the full potential use of its cellular machinery. On the neutrophil surface, a conformation change of constitutively displayed surface integrins takes place, while additional integrin adhesion molecules stored in secretory vesicles may also mobilize to the surface. Neutrophil integrins including CD11b bind ligands such as endothelial ICAM-1 and mediate firm adhesion halting the cell. Then, neutrophil begins MAC1-dependent crawling along the chemotactic gradient on the endothelium looking for a suitable site to transmigrate. Transmigration is preferentially paracellular—that is, at the endothelial cell–cell junctions. The transcellular route through endothelial cells may also be employed. This mode of transmigration is more time-consuming despite endothelial cells forming transmigratory domes, thus facilitating the process (Figure 3). After transmigration, neutrophils must cross the basement membrane, a process where granule-contained MMPs become relevant (*Delclaux et al. 1996*).

Figure 3.

Updated classic neutrophil recruitment cascade.



Adapted from Kolaczowska E *et al.* "Neutrophil recruitment and function in health and inflammation". *Nature Reviews Immunology* 2013; 13:159–175. Reprinted with permission from the copyright holder.

Once transmigration is achieved, three potential actions are open to neutrophils, as reviewed by Nathan (2006).

First, crawling along the concentration gradient of the chemotactic stimulus, neutrophils attempt to find, bind to, and initiate phagocytosis, thereby killing microbial targets (Roos *et al.* 2003). If successful, they continue until sufficiently clearing infectious organisms, then they can shift towards shutting off inflammation and promoting replicative wound healing.

Second, if neutrophils cannot make contact with consumable microbes sufficiently quickly but still sense inflammatory or infectious surroundings, they release their toxic armament upon degranulation and attempt to kill the intruder at a distance. In this all-out kamikaze response,

even their nuclear material remains behind as a lysozyme-decorated, bacteria-snaring neutrophil extracellular trap (*Brinkmann & Zychlinsky 2007*). In this mode, the potential for substantial host tissue damage exists, since destructive proteolytic enzymes are released freely. In essence, this type of inflammatory activity leads to the formation of pus, the liquefying properties of which are well known.

Third, if neutrophils do not find consumable microbes and do not sense further signs of danger, they identify the incident as a false alarm and undergo apoptosis.

The modes of action described above include exceptions, however. Recently, confocal intravital microscopy in the context of I/R injury identified the reverse transmigration of neutrophils *in vivo* (*Woodfin et al. 2011*). Furthermore, those results suggested that neutrophils reverse-transmigrating from inflamed tissue to the vessel lumen might contribute to the dissemination of systemic inflammation and injury to secondary organs.

Lactoferrin

Lactoferrin is a transferrin family multifunctional protein found in bodily fluids such as human milk, blood plasma, tears, saliva, and nasal secretions. As implied by its name, lactoferrin transfers iron to cells and, in part, regulates the concentration of free iron in bodily fluids. In addition, lactoferrin exhibits considerable antimicrobial activity. Its capacity to limit bacterial growth has been thoroughly studied, and relates in part to iron deprivation from growing bacterial cells (*Drago-Serrano et al. 2017*). Binding to specific receptors along the outer bacterial cell membrane provides another defensive mechanism, since in this bound form lactoferrin can promote bacterial oxidization via the formation of peroxide. Membrane-bound lactoferrin also opsonizes bacteria for phagocytosis (*Farnaud & Evans 2003*). The proteolysis of lactoferrin appears to yield stable antimicrobial peptides potentially exceeding the potency of intact lactoferrin (*Saito et al. 1991*). The antimicrobial activity of peptide fragments seem unrelated to iron chelation. In addition, the antifungal and antiviral properties are demonstrably distinct from the antibacterial activity of lactoferrin.

In neutrophils, specific granules contain lactoferrin. As a one facet of neutrophil activation, degranulation process occurs, whereby lactoferrin is released to the cell's surroundings (*Faurschou & Borregaard 2003*). Thus, lactoferrin readily detectable in blood can serve as an index of upstream neutrophil activity.

Matrix metalloproteinase 9 (MMP-9)

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases capable of breaking down components of the extracellular matrix (*Viappiani et al. 2006*). As such, MMPs have been extensively investigated given their involvement in numerous physiological and pathological processes. For instance, they play a role in extracellular matrix homeostasis, the spread of neoplastic tumors and metastases, the migration of inflammatory cells (*Verollet et al. 2011*), and the control of inflammatory vascular permeability. Neutrophils contain a readily available source of MMP-9 in their secondary and tertiary granules (*Wang et al. 2005*). Actively secreted MMP-9 represents a major factor in trans-basement membrane neutrophil migration (*Delclaux et al. 1996*). Renal MMP-9 appears to increase during experimental renal ischemia (*Caron et al. 2005, Caron et al. 2005, Bellini et al. 2007, Basile et al. 2004*). By destroying the extracellular matrix, MMP-9 may increase microvascular leakage and further enhance the extravasation of neutrophils during renal reperfusion injury (*Sutton et al. 2005*). Indeed, MMP inhibition provides protection from experimental renal I/R injury (*Kelly et al. 2004, Wang et al. 2005, Novak et al. 2010*). However, evidence also exists suggesting that processes leading to the reoxygenation of hypoxic tissues may depend on MMP-9 delivery via neutrophils (*Christoffersson et al. 2012*).

Tissue inhibitor of matrix metalloproteinase 1 (TIMP-1)

The tissue inhibitor of matrix metalloproteinase 1 (TIMP-1) belongs to the family of physiological tissue inhibitors of matrix metalloproteinases (TIMPs). TIMP-1 inhibits MMP activity by blocking the MMP catalytic core. Persistently elevated levels of TIMPs have been described in the context of renal transplantation, possibly triggered by the donation or implantation processes. The resulting diminished MMP activity, in turn, impacts the development of chronic allograft nephropathy (*Ahmed et al. 2012*).

Monocytes/macrophages

Monocytes are blood-borne macrophages and, as such, are relatively inactive. They travel to the target host tissue, where they differentiate into tissue-resident macrophages and adopt their phenotypic form, which is highly variable depending on the host tissue. In addition, macrophages are versatile cells with considerable longevity compared to neutrophils, capable of surviving for months at best. Macrophages take on the role of removing necrotic cellular debris or worn out cells (including neutrophils). They are also capable of functioning as antigen-presenting cells and, thus, play a key role alongside dendritic cells in initiating adaptive immune responses. Stimulated by T-lymphocyte-derived lymphokines, macrophages can also further differentiate into a more aggressive form ("armed macrophage") and protect against malignantly transformed cells or cells infected with fungi or parasites. Finally,

macrophages shut off inflammatory responses and participate in wound healing and tissue repair.

Complement system

Three different pathways activate the complement system, all of which converge in the formation of C3 and, subsequently, the membrane attack complex (MAC). The complement system provides an important link between the innate and adaptive immunity arms since its activation via the classical pathway involves the binding of an antibody. The role of the complement system in the humoral rejection of transplantations has been extensively described, while less is known about its interaction with T-cell cytotoxic responses. Growing interest in I/R injury control revealed the importance of the complement system in this respect as well (*Carroll et al. 2005, Zhang & Carroll 2007*). In renal transplantation-related I/R injury, complementary activation via an alternate pathway and the lectin-binding pathway appear more important than activation via the classic pathway (*Damman et al. 2008*).

COAGULATION AND HEMOSTASIS

Maintaining blood fluidity depends on the activity of the endothelial cells. As a boundary layer between blood and the parenchymal cells, endothelium also plays a key role in the recruitment of inflammatory cells. The endothelial cells comprise an “organ”, which weighs about 720 g in an adult, with more than 600 g of this cell mass dedicated to the capillaries. The overall endothelial surface area has been estimated at 3000 to 6000 m² (*van Hinsberg 2012*).

The obvious goal of blood coagulation is achieving hemostasis after an injury to a blood vessel. When a vascular wall breach occurs, blood elements come into contact with structures not encountered within an intact vasculature. The most notable structures include collagen and tissue factor. Platelets bind collagen via specific receptors and aggregate together to form a platelet plug. Simultaneously, activated platelets and tissue factor exposed from the subendothelial space trigger a coagulation cascade culminating in the formation of fibrin, which serves to further stabilize the clot. These processes occur simultaneously and are termed primary and secondary hemostasis, respectively. The process of fibrinolysis concomitantly initiates and represents the resolution phase as the original violation is controlled.

Primary hemostasis

Primary hemostasis results in the formation of a platelet plug. When the vascular wall is compromised, extracellular matrix elements are exposed to blood-borne cellular and acellular components. Platelets bind to the exposed extracellular matrix through a multitude of adhesive molecules, including their glycoprotein receptors that bind collagen. The platelet functional activation leading to the further recruitment of additional platelets and the activation of the coagulation cascade are complemented by the changing shape of the platelets from spherical to stellate.

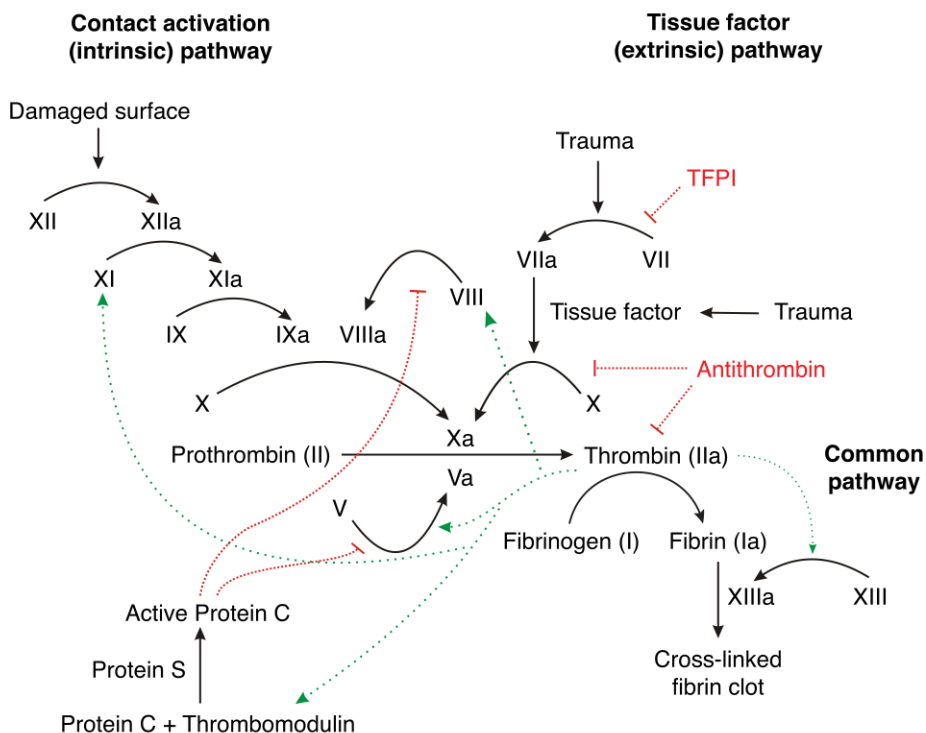
Secondary hemostasis

The coagulation cascade related to secondary hemostasis consists of a series of enzyme precursors that circulate freely in the blood stream, typically called coagulation factors. As the cascade is triggered, the active form of the coagulation factor catalyzes the activation of the next factor in the series, resulting in a spatially controlled reaction amplification, thus creating the so-called thrombin burst and ultimately forming insoluble fibrin. The cascade encompasses both negative and positive feedback loops that prevent the uncontrolled spread of activation throughout the vasculature.

In short, when the endothelial disruption triggers the tissue factor (TF) pathway, factor VII makes contact with TF, yielding the activated complex of TF and FVIIa. The TF–FVIIa complex activates FIX and FX. FXa and FVa aggregate to form a prothrombinase complex, which then catalyzes prothrombin conversion to thrombin. The creation of thrombin in large enough quantities is essential, since thrombin catalyzes the final step of cleaving fibrinogen to insoluble fibrin, thus reinforcing the platelet plug of primary hemostasis. Thrombin also represents the most important site of feedback regulation for the coagulation cascade (described below). Figure 4 provides a schematic representation with some of the regulatory elements shown.

Figure 4.

Blood coagulation cascade.



Adapted from Joe Dunckley / Wikipedia.

The newer cell-based model of coagulation expands the cascade model encompassing a multitude of cellular elements that inevitably participate in coagulation processes *in vivo*. Several bleeding or thrombotic clinical disorders remain theoretically unexplained, if the impact of phospholipid bilayers remains unconsidered. Today, the presence of polarized phospholipid bilayers and incorporated proteins represent a proven regulatory element in coagulation (*Smith 2009*).

Aside from its central role in blood coagulation, thrombin is also recognized as one of the factors contributing to perioperative inflammatory syndromes (*Raivio et al. 2006, Untch et al. 2008*). Prothrombin fragment F1+2 is a byproduct of the cleavage of prothrombin to thrombin readily detectable in blood. Thus, it can be used as an indicator of the ongoing thrombin formation.

Endogenous anticoagulation

Activated protein C (APC) performs widespread functions in regulating blood coagulation and inflammation. Protein C (PC) is a K vitamin-dependent glycoprotein that circulates in the blood in the zymogenic or inactive form. Activation takes place upon binding to thrombin and is amplified by complexation with thrombomodulin and the endothelial protein C receptor (EPCR) on the endothelial surfaces. In the active form, APC exerts its anticoagulant properties through the proteolytic inactivation of coagulation factors V and VIII. As such, PC-APC is regarded as the most important naturally occurring anticoagulant in humans, whereby its clinical importance is highlighted by the fatal course of neonatal protein C deficiency when left untreated (*Esmon & Schwarz 1995*).

The tissue factor pathway inhibitor (TFPI) is another major physiological regulator of TF-induced blood coagulation. TFPI affects coagulation by directly inhibiting factor Xa and by the FXa-dependent inhibition of the TF-FVIIa complex. In this interaction, protein S works as a cofactor in the direct inhibition of FXa (*Ellery & Adams 2014*).

Antithrombin represents yet another major endogenous anticoagulant, which degrades several coagulation factors including thrombin, FIXa, FXa, FXIa, and FXIIa. Its actions are exploited clinically, since an affinity towards the antithrombin substrates can be enhanced quite substantially using different heparin preparations.

Fibrinolysis

As soon as some fibrin has been deposited within the vascular network, concurrent fibrinolysis occurs, aimed at protecting against excessive coagulation and allowing for revascularization. To this end, the liver synthesizes plasminogen, of which plasmin is created

via proteolytic cleavage by the endothelium-synthesized tissue plasminogen activator (tPA). Plasmin cleaves fibrin into fibrin degradation products.

D-dimer is a small soluble fibrin degradation product found in the blood. The laboratory determination of D-dimer enjoys wide clinical use as a marker for increased fibrin turnover. The possible effects on inflammation, however, remain poorly understood. Yet, for other fibrin fragments, such fibrin fragment E, some evidence of a pro-inflammatory activity exists. The impact of these fragments *in vivo* is yet to be determined (*Jennewein et al. 2011*).

Along with plasmin, the **tissue plasminogen activator (tPA)** is a key enzyme in fibrinolysis and blood clot dissolution. Produced primarily by endothelial cells, tPA functions by converting blood-borne inactive proenzyme plasminogen into active serine protease plasmin, which, in turn, degrades fibrin. Normally, only minute concentrations of tPA circulate. Under normal circumstances, tPA endothelial secretion falls under complex hormonal and physical factor controls. The enzymatic activity of tPA greatly increases in the presence of fibrin, thereby localizing the fibrin-degrading activity of plasmin on fibrin surfaces (*Loscalzo 1988*). Experimental evidence points towards the role of tPA in neutrophil trafficking in renal I/R injury, potentially representing a target for therapeutic intervention (*Roelofs et al. 2006*).

Plasminogen activator inhibitor 1 (PAI-1) plays an important role in maintaining the delicate equilibrium between hemostasis and blood clot dissolution (*Brown 2010*). PAI-1 is a serine protease inhibitor functioning as the principal inhibitor of tPA. The main site of PAI-1 production is the endothelium, although other sources have also been recognized. Along with tPA, MMP actions are also inhibited by PAI-1 (*Ha et al. 2009*).

INFLAMMATION

Inflammation is the body's response to tissue injury, whether from an infectious etiology or not. Because multicellular organisms have faced constant microbial challenges for several hundred million years, quite potent protective systems have evolved. However, since the fundamentals of biochemistry are preserved through the evolution of all genetic material-containing organisms, the machinery used to attack and kill an invader also carries a considerable potential to harm the host as well. This dictates the need to maintain continuous homeostatic control over such mechanisms, only permitting activation in a strictly controlled manner, both spatially and temporally (*Nathan 2006*). In the wild or in nature, a tissue injury without concomitant microbial infection or at least contamination is virtually impossible. In the modern world, however, injury without infection is possible to some extent in the unnatural environment of a sterile operating theater.

Histologically acute inflammation is often characterized by tissue neutrophil infiltration. Neutrophils are followed by blood monocytes that mature into inflammatory macrophages. Resolution is characterized by the clearance of neutrophils and the tissue mononuclear cell population's return to a normal pre-inflammatory state.

As neutrophils commit to an inflammatory action, several security checkpoints need to be passed. These involve contact with pro-inflammatory molecules, pathogens, or DAMPs as well as cellular contact including activated endothelium, activated lymphocytes, and activated macrophages or dendritic cells (*Nathan 1987*). When these prerequisites are met, neutrophils are activated and their longevity is dramatically increased. Having adopted a pro-inflammatory phenotype, neutrophils also recruit more leukocytes to the scene. Recent evidence suggests that among the neutrophil population, different subsets with varied patterns of cytokine secretion may exist and these subsets may differ in their expression of TLRs and integrins. Experimental data indicates that differing neutrophil subsets play a role in shaping the course of the immunological response depending on the type of challenge at hand (*Kolaczowska & Kubes 2013*).

Just as mobilizing and recruiting inflammatory armamentum quickly when needed is important, cessation and resolution must also promptly follow in order to avoid host-tissue destruction (*Nathan 2002*). A body of evidence suggests that immediately during the beginning phases of acute inflammation, a preprogrammed end to the inflammatory process is initiated. The biochemical basis for this may lie in the regulation of eicosanoid family bioactive lipid mediators, so that a shift from the synthesis of pro-inflammatory prostaglandins to the synthesis of resolution and anti-inflammation promoting lipid mediators takes place. In this context, new classes of mediators—lipoxins, resolvins, and protectins—have been identified (*Serhan & Savill 2005, Serhan 2008*).

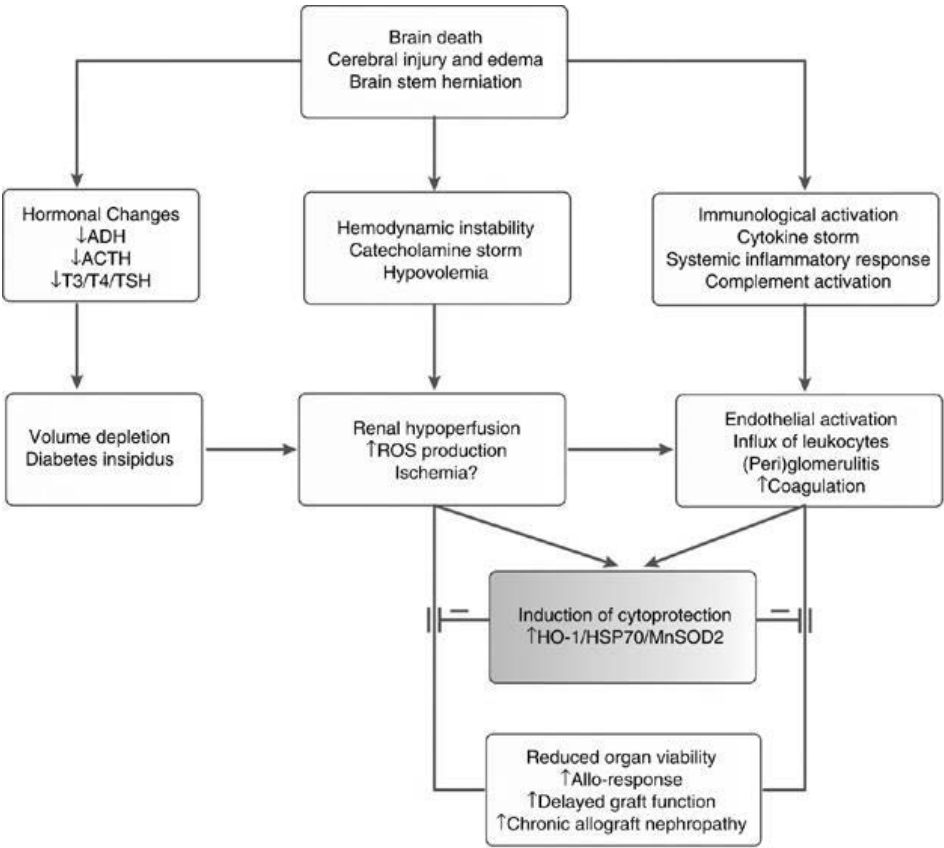
Due to the many intruder shapes and forms, a tremendous amount of redundancy and overlapping mechanisms of mammalian inflammatory and immune systems has evolved, of which the abovementioned only touch upon briefly. Hence, pursuing successful whole organ transplantation, clinicians face the formidable tasks of simultaneously inhibiting any inflammatory injury to the graft and protecting the graft and recipient from infectious risks. Inflammatory system redundancy renders escaping detection difficult for pathogens, while also making it difficult for transplantation physicians to shut down these systems safely, even for a short while, in order to perform implantation surgery.

Donor inflammatory response

Brain death induces a profound inflammatory response in the donor as well as serious perturbations in other homeostatic systems, including the blood pressure and hormonal control. These can be managed to a certain degree, but typically require intensive care as well as technical resources and personnel (*Westendorp et al. 2011*). Some claim that managing brain-dead donors is the most neglected area of transplantation medicine.

Contrary to outside perceptions, brain death is not a static condition (*Bos et al. 2007*). Cerebral injury or trauma is followed by an increase in intracranial pressure with immediate hormonal changes. The increasing intracranial pressure triggers parasympathetic activity, resulting in systemic hypotension. Hypoperfusion and ischemia of the peripheral organs may also occur at this stage (Figure 5). When the brain stem is herniated, sympathetic stimulation together with persistent parasympathetic activation causes further disturbances, including bradycardia, hypertension, and an irregular breathing pattern. Collectively, as early as 1902 these disturbances were labeled the Cushing reflex (*Cushing 1902*). When the vagal cardiomotor nucleus becomes affected, solitary sympathetic stimulation supervenes. At this stage, a massive systemic release of catecholamine hormones occurs. This “catecholamine storm” then leads to a hypertensive crisis. Intense vasoconstriction in the peripheral vascular beds, in turn, can lead to end organ–level parenchymal hypoxemia. Experimentally catecholamine-induced vasoconstriction may cause a dramatic decrease in the renal blood flow due to an increase in vascular resistance (*Herijgers et al. 1996*). All tissues suffering from oxygen deprivation rely on anaerobic energy metabolism, which, in turn, leads to the production of lactate and anaerobic energy metabolism intermediates. The accumulation of these products leads to later vasodilation, which then in conjunction with other perturbations (e.g., heart failure) may cause hemodynamic collapse. This subjects peripheral tissues to further oxygen deprivation creating a vicious circle.

Figure 5.
Proposed model for the (patho)-physiological changes associated with brain death.



Adapted from Bos EM *et al.* "Kidney grafts from brain dead donors: Inferior quality or opportunity for improvement." *Kidney Int* 2007; 72:797–805. Reprinted with permission from the copyright holder.

Experimental studies on brain death have pinpointed pituitary failure as a source of major hormonal perturbations. In humans, however, data are scarcer. Somewhat better understanding exists regarding the appearance of diabetes insipidus as antidiuretic hormone (ADH) production diminishes. The resulting polyuria contributes to hypovolemia and problems maintaining an adequate perfusion pressure (*Chen et al. 1999*). Treatment with thyroid hormones, ADH, and insulin as well as the use of corticosteroids is often deemed necessary, although the resulting clinical outcomes appear somewhat inconclusive.

Growing evidence indicates that systemic inflammatory changes following brain death occur (Takada et al. 1998). The primary reason behind systemic inflammation remains unclear, however. Possible sources for the observed elevation in inflammatory mediators in the blood stream include an ischemic brain, an ischemic intestine, bouts of anaerobic metabolism, extreme swings of blood pressure, poor control of blood glycemia, and the release of peripheral neuropeptides no longer under cortical control (Floerchinger et al. 2012).

Among other inflammatory responses, brain death has also emerged as an important inducer of complement activation. The data on the complement system's role in renal I/R injury remain somewhat contradictory, however. Admittedly, experimental interventions targeting various complement components have proven protective against renal I/R injury. However, it is also noteworthy that the kidney itself has emerged as a site of marked complement production and many renal cellular structures exhibit a complement-producing capacity *in vitro* (Jager et al. 2017).

Today, the end-organ effects of brain death are not understood in detail in humans, but direct evidence of donor kidney injury has been demonstrated. Compared to living donor kidneys, differences in leukocyte infiltration, endothelial adhesion molecules, and inflammatory gene expressions are observed (Nijboer et al. 2004). The mechanisms behind clinical complement-related injury are currently being examined (van Werkhoven et al. 2013).

Ischemia–reperfusion (I/R) injury

Persistent ischemia of the tissue induces cellular responses. On the one hand, these responses work to eliminate (microbial) invaders and restore oxygenation by activating inflammatory responses and, on the other hand, attempt to help cells adapt to and withstand hypoxia through anti-inflammatory, anti-apoptotic, and regenerative responses. The cellular oxygen-sensing signaling pathway of prolyl hydroxylases (PHD) and the hypoxia-inducible factor (HIF) lie at the heart of hypoxia-induced responses (Eltzschig & Carmeliet 2011). Examples of PHD–HIF pathway pro-inflammatory signaling include the transcriptional promotion of the pro-inflammatory nuclear factor kappa B (NF-κB) and TLRs, and this pathway also impacts human renal transplantation (Rosenberger 2007). One important result stems from the conversion of the endothelium from the anti-adhesive and anticoagulation phenotype to the adhesive and procoagulant phenotype. The capability of the PHD–HIF pathway to help withstand hypoxic milieu, then, is in part mediated by the transcriptional promotion of heat-shock proteins. The abovementioned examples involve many important messenger molecules and intermediates such as adenosine, netrins, and sphingolipids (Rosenberger et al. 2009, Bartels et al. 2014). Altogether, cellular hypoxia signaling has proven to be a very complex process and intensifying research has identified new regulatory elements (Furuichi et al. 2008). Obviously,

the field carries significant translational relevance since ischemia-protective pharmacological interventions remain insufficient and injury prophylaxis rather than treatment would appear advantageous (*Eltzschig & Eckle 2011*).

When perfusion is regained following a period of ischemia, paradoxically tissue injury exacerbation and profound inflammatory responses tend to occur. Many overlapping mechanisms may contribute to this paradox. As blood flow is reinstituted, a low oxygen milieu is suddenly reversed to a high oxygen milieu. This favors the creation of reactive oxygen species (ROS), highly reactive molecules capable of inflicting, for instance, cellular protein, as well as lipid and DNA damage (*Kosieradzki & Rowiński 2008*). The NADPH oxidase enzyme, expressed by virtually all inflammatory cells, may generate cytotoxic ROS peroxynitrite. In addition, H₂O₂ derived from O₂ dismutation, in turn, results in toxic hydroxyl radicals via a Haber–Weiss reaction, facilitated by the increased availability of free iron in ischemia (*Kehrer 2000*).

Due to the ischemia-induced inflammatory signaling activity, the accumulation of inflammatory cells follows the restoration of blood flow. Innate immune cells dominate the cellular infiltrate composition during the early phases of reperfusion. The contributions of separate cell types to the ensuing tissue damage are not unambiguous. For example, recruited monocytes appear to beneficially participate in the healing processes after an ischemic injury. Neutrophils, by contrast, are often detrimental since their vast numbers and cellular makeup carry significant destructive potential. However, it is also possible that too few neutrophils may not allow for the adequate clearance of debris and subsequent tissue repair, with potential negative implications particularly in the context of transplantation, where alloantigens are present. In any case, infiltrating cells exhibit pro-inflammatory phenotypes including a heightened cytokine production and the upregulation of TLRs, of which TLR4 specifically has been studied within this context (*Jang & Rabb 2009*).

I/R injury also initiates a considerable adaptive immune response (*Boros & Bromberg 2006*). Lymphocytic cellular infiltrates play different roles in I/R injury (*Linfert et al. 2009*). CD4+CD8+ T cells appear to carry detrimental roles in various ischemia reperfusion settings. T-reg cells, by contrast, attenuate I/R injury in experimental settings. Dendritic cell recruitment represents one of the major immune responses associated with transplantation-related I/R injury and this type of response accompanies even syngeneic transplantations (*Schlichting et al. 2006*).

In addition, subcellular elements may contribute to I/R injury. Platelet aggregation and activation yields pro-inflammatory and procoagulant products and, in part, participates in complement activation. Complement activation, in turn, may propagate due to the recognition of ischemia-induced neoepitopes via naturally occurring antibodies. Complement activation then leads to further neutrophil recruitment and tissue injury.

Ischemia and reperfusion in transplantation

Organ ischemia of some duration is inevitable in deceased-donor transplantation. In addition to physical cooling, several preservation solutions are employed to preserve organ viability (Wilson *et al.* 2006). Common to these solutions is their intracellular compartment-like electrolyte content aimed at diminishing cell swelling caused by the cell membrane ion pumps progressively ceasing their action when adenosine triphosphate (ATP) is depleted. A buffering capacity is also added in order to counter lactate creation, since cells rely on anaerobic energy metabolism during preservation (Lee & Mangino 2009). Recent organ preservation advances include machine perfusion methods with or without hypothermia. The feasibility of these methods remains to be determined, however (Timsit & Tullius 2011).

Several strategies in solid organ transplantation contexts have been investigated in order to prevent the pathological chain of events described above. These include ischemic preconditioning, leukocyte depletion (Sievert 2003), manipulation of complement activation, manipulation of inflammatory signaling, adhesion molecule blockade, and the administration of antioxidants. These interventions all exhibit some benefit, particularly in experimental settings, whereby clinical trials often result in disappointing results. As such, none of these interventions have been widely adopted thus far.

No reflow phenomenon

The no reflow phenomenon refers to a state where macro- or microvascular blood flow is not effectively restored after declamping of the allograft vasculature. Difficulties in the reflow of the larger vessels result from errors in surgical technique and clot formation due to inadequate flushing during procurement. In addition, *de novo* thrombosis may occur particularly in recipients with undiagnosed hypercoagulation disorders.

At the microvascular level, differing patterns of the no reflow phenomenon may be observed. The phenomenon may occur in a more or less regional manner (Yamamoto *et al.* 2002). Capillary no reflow may result from microthrombosis or endothelial blebbing, impaired vasorelaxation, and capillary plugging due to leukocytes (Snoeijs *et al.* 2010). During tissue inflammation, leukocyte trafficking normally takes place in the postcapillary venules. These vascular sites are well suited to trafficking for several reasons. Firstly, the blood flow in the postcapillary venules is slow. This results in red cells forming columns or *rouleaux*, which then populate the axial center of the blood vessel lumen, pushing the white cells to the sides and into contact with the endothelium. Secondly, because postcapillary venules are also sites for endothelial adhesion molecule presentation, white cell rolling, firm adhesion, and transmigration can effectively take place. Thirdly, postcapillary venules have a sufficient

diameter enabling the maintenance of forward blood flow even in situations where substantial endothelial surfaces become involved in the process of leukocyte margination and transmigration.

Alas, because tissue ischemia is prolonged, capillaries also begin expressing adhesion molecules (*Yamazaki et al. 1993*). This carries adverse consequences during reperfusion. Being pancake shaped, red cells can fold up upon themselves (like rolled crepes) and squeeze through the capillaries even when some endothelial swelling is present. The case is different for the spherical-shaped white cells, however. Even during normal capillary blood flow, white cells are sausage shaped when squeezing through capillaries and, thus, create a wide contact area with the endothelium. When endothelial cells are swollen or adhesion molecules are present, the white cells are effectively stopped inside the capillaries preventing further blood flow (*Engler et al. 1983*). When this happens, a progressively larger proportion of the blood flow is diverted upstream from capillaries, taking place through larger-diameter arteriovenous connections. Measured from the major graft-supplying artery, the blood flow may continue with little noticeable interference, although seriously jeopardized blood flow at the capillary level means prolonged tissue reliance on anaerobic metabolism.

Protein C: activated protein C (APC)

Aside from regulating blood coagulation, APC also possesses several anti-inflammatory properties exerted on the endothelial cells and leukocytes (*Levi et al. 2012*). These serve to downregulate the production of pro-inflammatory cytokines and to reduce the adhesion and activation of leukocytes. Anti-inflammatory effects largely depend on EPCR-bound APC signaling via protease-activated receptor 1 (PAR-1). Broadly, anti-inflammatory APC signal transduction cascades converge at the level of NF- κ B, the prototypical pro-inflammatory transcription factor (*Esmon 2012*). Local direct effects on the rate of neutrophil recruitment may be mediated in part by decreasing the thrombin-dependent mobilization of the Weibel–Palade bodies. In the endothelial cells, the Weibel–Palade bodies represent cytosolic storage granules rich in P-selectin, the von Willebrand factor, and angiopoietin-2. P-selectin is involved in leukocyte adhesion, while the von Willebrand factor contributes to platelet adhesion and angiopoietin-2 modulates endothelial permeability (*van Hinsberg 2012*).

AIMS OF THE STUDY

This thesis study aimed to investigate inflammatory and blood coagulation mechanisms during the immediate early reperfusion period of clinical renal transplantation. Due to the method of patient recruitment drawing from another interventional study, additional novel information concerning the intervention study pharmaceuticals was anticipated.

The specific aims of this research are as follows:

- I. To describe the early neutrophil activation and sequestration in clinical cadaveric renal transplantation and to explore the possible effects on clinical parameters and outcomes (study I).
- II. To describe the local and systemic response of the protein C-activated protein C axis in clinical renal transplantation (study II).
- III. To explore the activation of blood coagulation in the renal graft vasculature during early reperfusion and to investigate the possible clinical relevance (study III).
- IV. To describe the systemic and renal graft local kinetics of matrix metalloproteinase 9 in clinical renal transplantation reperfusion and to explore the possible clinical implications (study IV).

PATIENTS AND METHODS

PATIENTS AND STUDY DESIGN

For this study, patients were recruited from a larger efficacy and safety study conducted within the Helsinki University Hospital Transplantation and Liver Surgery Clinic between 1999 and 2001. Patients younger than 16 years, older than 65 years, with a malignant disease history, and with panel reactive antibodies (PRA) higher than 50% or who lost a previous graft within one year due to immunological reasons were excluded from the study. In addition, written informed consent was obtained before entering the study. A total of 168 patients were originally recruited, whereby 155 patients continued in the study and received a single 9-mg/kg dose of antithymocyte globulin (ATG)-Fresenius (group A) or two 20-mg doses of basiliximab with a reduced dose of cyclosporin A (CsA) (group B) or a conventional CsA triple therapy without induction (group C) (*Kyllönen et al. 2007*). The local ethics committee of the surgical hospital at Helsinki University Hospital (HUH) approved the amendment to the study plan permitting additional blood sampling for this thesis project. A total of 45 patients participated in the additional analyses comprising the research in this thesis project, retaining the original randomization: group A (n = 15), group B (n = 16), and group C (n = 14).

The administration of the different immunosuppressive agents was completed before commencing graft reperfusion.

Laboratory analyses were conducted in the Department of Bacteriology and Immunology at Haartman Institute at the University of Helsinki (studies I, III, and IV), in the Department of Clinical Chemistry at HUCH and the Helsinki University Laboratory (HUSLAB) (studies I–IV), and in the Department of Molecular and Experimental Medicine, Scripps Research Institute (La Jolla, CA, USA) (study II).

CLINICAL PATIENT DATA

Donors

All renal grafts were procured from brain-dead heart-beating donors. The donor age was recorded, and no differences existed between groups based on age.

Recipients

Among recipients, age, gender, medication, etiology of the terminal uremia, weight, and height were recorded.

RESEARCH SAMPLES

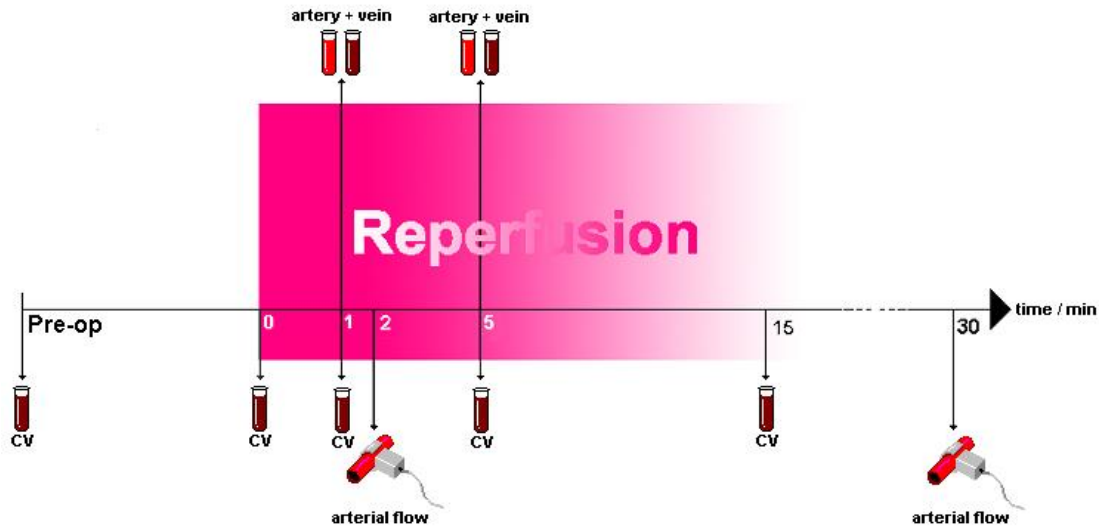
Blood sampling and blood volume flowmetry

For each patient, a preoperative and a pre-reperfusion blood sample was drawn from the central venous cannula. The preoperative sample was obtained after induction of anesthesia, but before commencing with the surgical procedure. The pre-reperfusion sample was obtained after the completion of vessel anastomoses, but before declamping of the iliac artery and proximal iliac vein. Paired local reperfusion blood samples were obtained at 1 and 5 min after reperfusion from the graft supplying artery and vein (i.e., from the recipient's iliac artery proximal to the anastomosis and from the renal vein of the graft, respectively). Samples were obtained via direct intraluminal needle aspiration by the operating and assisting surgeons. Along with the paired directly aspirated samples, the anesthesiologist also drew central venous samples. Finally, a 15-min post-reperfusion sample was drawn from the central venous cannula. Postoperative cubital vein blood samples were obtained at postoperative days 1, 2, 4, and 6. The volume of each blood sample was 10 ml, and samples were drawn into pyrogen-free plastic disposable syringes and immediately transferred to pyrogen-free test tubes. From these tubes, aliquots were further transferred into prechilled test tubes according to the particular laboratory method used (discussed below).

Arterial blood flow was measured by the operating surgeon at 2- and 30-min post-reperfusion using a commercial ultrasound transit-time flowmeter (Cardiomed Volume Flowmeter, Medi-Stim AS, Oslo, Norway). If the graft consisted of multiple arteries, all were measured and the sum was used for statistical analysis. The blood sampling and arterial blood flow measurement schema are summarized in Figure 6.

Figure 6.

Schema for the blood sampling and flow measurements.



LABORATORY METHODS

Leukocyte counts (studies I and II)

Leukocyte differential counts were obtained using a Technicon H2 hematology analyzer (Bayer Corporation, Tarrytown, NY, USA) operated by Helsinki University Clinic laboratory personnel.

Adhesion molecules CD11b and L-selectin (studies I and II)

Adhesion molecule CD11b and L-selectin (CD62L) expression on circulating neutrophils and monocytes were analyzed by means of flow cytometry. The corresponding cell labeling was carried out in a +0°C ice-water bath with ice-cold reagents. For each blood sample, a 1-ml aliquot was transferred into a prechilled polystyrene tube (Falcon No. 2058 Becton–Dickinson Labware, Lincoln Park, NJ, USA) supplemented with 1 ml of an ACD solution (pyrogen-free citrate, Baxter Healthcare, Norfolk, UK; Baxter product no. 93I08BH). The tube was kept in a styrofoam box containing 0°C water and ice until labeling. Sample preparation and cell double labeling were carried out as described elsewhere (*Repo et al. 1993, Repo et al. 1995*). Briefly,

for neutrophils, 25- μ l aliquots of blood were labeled within 24 h of sampling with fluorescent anti-CD11b-phycoerythrin (PE) and anti-CD62L-fluorescein isothiocyanate (FITC) antibodies (BD Biosciences, San Jose, California, USA) and incubated in the dark at 4°C. Erythrocytes were lysed with FACS lysing solution (BENEX Limited, BD Biosciences, Shannon, County Clare, Ireland) and leukocytes were collected by centrifugation at 4°C. Labeled cells were resuspended in 1% formalin and kept at 4°C until analysis. Similarly, for monocytes, two 25- μ l aliquots were labeled with anti-CD11b-PE and anti-CD14-FITC or anti-CD62L-FITC and anti-CD14-PE. Data acquisition and analysis were completed using a FACSort flow cytometer and CellQuest Pro analysis software (both from BD Biosciences, San Jose, California, USA). Neutrophils were identified by their light scattering pattern, while monocytes were identified by their light scattering pattern and CD14 positivity. Adhesion molecule expressions are reported in relative fluorescence units (RFUs)—that is, as the mean channel of the positive-fluorescing cell population.

Lactoferrin (study I)

Lactoferrin was measured using enzyme-linked immunosorbent assay (ELISA) by anti-lactoferrin antiserum (Dako, Glostrup, Denmark), purified in a lactoferrin-Sepharose (Amersham Pharmacia Biotech, Uppsala, Sweden) affinity column and labeled with alkaline phosphatase. Microtiter plates were coated with 5- μ g/mL affinity-purified antibodies. Purified human lactoferrin (Sigma Chemical Co., St. Louis, MO, USA) was used as the standard, and the standards and samples were diluted to cover a range of 5 to 100 ng/mL.

Protein C and activated protein C (study II)

The plasma levels of protein C and APC were determined using an enzyme capture assay as previously described (*Gruber & Griffin 1992*). Briefly, a monoclonal antibody against protein C was immobilized on microplates, and the surface was blocked. Plasma samples supplemented with benzamidine, a reversible inhibitor of thrombin, APC, and trypsin-like proteases were incubated in the wells to capture APC and the protein C antigen. Then, the plates were washed to remove sample constituents and benzamidine. The amidolytic activity of the captured APC was measured using the chromogenic substrate S-2366 (Chromogenix AB, Mölndal, Sweden). Assays were run in duplicate, and a noncommercial plasma pool was used as the standard. The sensitivity of the assay was set to 5 pmol/L, corresponding to 13% of the normal mean plasma level of APC.

Total protein C was measured by activating the bound protein C in the immunocaptured samples using a snake venom activator enzyme (Protac, American Diagnostica, Greenwich,

CN, USA), followed by measurement of the amidolytic activity on a chromogenic substrate S-2366. Since APC was less than 1% of the total protein C, the amidolytic activity observed after Protac activation essentially equaled the total protein C level. Assays were run in duplicate, and pooled normal human plasma (Precision Biologicals, Dartmouth, Nova Scotia, Canada) was used as the standard. The results of APC and the total protein C are expressed as a percentage relative to the plasma pool defined as 100%.

Coagulation parameters (study III)

All coagulation parameters were assayed using commercial ELISA kits following the manufacturer's instructions, including the following: prothrombin fragment F1+2 (Enzygnost F1.2micro, Dade Behring, Liederbach, Germany); tPA antigen and tPA activity (t-PA Actibind, Technoclone, Vienna, Austria); PAI-1 (PAI-1 Antigen ELISA, Technoclone, Vienna, Austria); and D-dimer (IMUCLONE D-Dimer ELISA, American Diagnostica, Stamford, CT, USA).

MMP-9 and TIMP-1 (study IV)

Extracellular protease and protease-inhibitor parameters were assayed using commercial ELISA kits following the manufacturer's instructions (Biotrak, Amersham Pharmacia Biotech, Little Chalfont, UK).

STATISTICAL ANALYSIS

Statistical analyses were carried out using various versions of SPSS software for Microsoft Windows (versions 9–20, SPSS Inc., Chicago, IL, USA). The non-parametric Wilcoxon test for paired samples was used to test the significance of the transrenal changes across parameters (studies I–IV). The Mann–Whitney U test (studies I–IV) and binary logistic regression (study I) were used for the analyses between groups. Multiple linear regression analysis was used to evaluate the relative importance of explanatory variables on the observed behavior of the variable of interest (study II). The Pearson product–moment correlation coefficient was determined to explore linear correlations between variables (studies I, II, and IV). The receiver operator characteristic (ROC) analysis was used to test the predictive value of parameters (study IV).

Since ATG administration resulted in quite marked changes for some of the parameters studied, excluding group A patients and/or a combination of group B and C patients as a larger combined BC group was done for some of the statistical analyses, as indicated in the original publications (studies I, III, and IV). Patient data are presented as medians and range.

RESULTS

PATIENT AND CLINICAL OUTCOME DATA

Cold and warm ischemia times did not differ between groups. The median cold ischemia times were as follows: group A, 24.0 h; group B, 24.8 h; and group C, 21.6 h. In comparison, the warm ischemia times were 0.74 h for group A, 0.65 h for group B, and 0.81 h for group C. Table 2 summarizes the characteristics for the clinical recipients.

Table 2.

Patient data.

Group	A	B	C
Age	51 (24-62)	44 (26-61)	54 (29-64)
Sex (M/F)	10/5	6/10	8/6
Diagnosis			
Diabetic nephropathy	5	5	4
Polycystic renal disease	4	2	4
Chronic glomerulonephritis	2	2	0
IgA nephropathy	2	0	1
Chronic pyelonephritis	0	1	2
Other	2	6	3

Adapted from Turunen AJ *et al.* Association of graft neutrophil sequestration with delayed graft function in clinical renal transplantation. *Transplantation* 2004; 77:1821-1826. Reprinted with permission from the copyright holder.

One patient developed acute myocardial infarction during the postoperative phase and died with a well-functioning kidney graft on day 10 post-transplantation. One graft was lost on day 69 post-transplantation, having regained only marginal functioning for a period of time following transplantation. Another graft was lost to the recurrence of focal segmental glomerulosclerosis on day 111 post-transplantation. One-year patient survival for the entire study population reached 97%, while graft survival was 93%.

Delayed graft function (DGF)

The overall incidence of DGF irrespective of study group was 29% (Table 3). In patients not treated with ATG, DGF associated with a longer cold ischemia time ($p = 0.036$).

Table 3.

Delayed graft function and the 1st month creatinine values by study groups

Group	A	B	C
Delayed graft function	3/15	5/16	5/14
Serum creatinine ($\mu\text{mol/L}$)			
6 th post op. day	145 (68-597)	152 (70-634)	129 (83-1144)
21 st post op day	130 (71-185)	109 (65-262)	121 (75-475)
30 th post op day	130 (83-179)	124 (75-278)	121 (89-368)

Adapted from Turunen AJ *et al.* Activated protein C reduces graft neutrophil activation in clinical renal transplantation. *American Journal of Transplantation* 2005; 5: 2204–2212. Reprinted with permission from the copyright holder.

SYSTEMIC INFLAMMATION AND COAGULATION RESPONSES

Patients receiving ATG (group A)

A single-dose perioperative administration of ATG resulted in profound alterations in virtually all of the parameters studied unparalleled by other induction schemes. In addition to the expected lymphocyte ablative effect of ATG, the numbers of circulating neutrophils and monocytes also significantly diminished. Thus, by the time of renal graft vascular declamping, circulating neutrophil numbers decreased to 27% of the values observed at the induction of anesthesia. Simultaneously and illustrating the relative cell lineage specificity of ATG, however, the circulating lymphocyte counts were as low as 4%.

Mirroring the cell depleting quality of ATG, a simultaneous increase in the phagocyte activation markers was observed, whereby the CD11b expression of neutrophils and monocytes roughly doubled. A concomitant twofold increase in systemic lactoferrin and up to

a tenfold increase in the MMP-9 concentration was also observed. Neutrophil L-selectin expression remained stable, however.

Across all study groups, the PC and APC levels fell within normal (e.g., 100%) levels at the beginning of surgery. Upon ATG administration, however, systemic APC levels doubled, while the elevation continued when reperfusion of the graft began. Correspondingly, among ATG-treated patients, systemic platelet counts decreased and F1+2, the tPA antigen, tPA activity, and the D-dimer levels increased significantly before reperfusion. In addition, D-dimer continued increasing following reperfusion. Table 4 summarizes the ATG effects.

Table 4.

ATG effects before surgery and reperfusion (*p<0.05 vs. Before operation).

	Before Operation	Before Reperfusion
Neutrophil count ($\times 10^9$)	3.15 (1.30–7.26)	0.85 (0.27–1.25)*
Monocyte count ($\times 10^9$)	0.48 (0.24–0.70)	0.03 (0.00–0.06)*
Lymphocyte count ($\times 10^9$)	1.49 (1.09–3.34)	0.07 (0.04–0.35)*
Platelet count	214 (109–374)	145 (71–283)*
Neutrophil Cd11b (RFU)	261 (113–379)	407 (180–688)*
Neutrophil L-selectin (RFU)	213 (109–272)	269 (172–310)*
Lactoferrin ($\mu\text{g/l}$)	92 (44–197)	195 (91–461)*
MMP-9 (ng/ml)	21 (7–43)	128 (76–286)*
TIMP-1 (ng/ml)	161 (90–299)	142 (94–197)*
APC (%)	119 (88–144)	232 (85–1246)*
F1+2	1.2 (0.4–2.8)	1.8 (0.4–3.4)*
tPA antigen	9 (1–25)	90 (29–138)*
D-dimer	281 (8–678)	624 (27–4278)*

Patients not receiving ATG (groups B and C)

Among patients not treated with ATG, only subtle changes in the phagocyte activation were observed in the systemic circulation. The systemic neutrophil CD11b expression decreased during the early stages of surgery, but remained stable after declamping of the graft vessels. The systemic neutrophil L-selectin expression mirrored changes to CD11b; as such, it increased before vascular declamping and remained stable thereafter (Table 5). The systemic lactoferrin concentration decreased slightly before reperfusion, but remained stable

thereafter. By contrast, the MMP-9 systemic concentration increased slightly before reperfusion and, then, remained stable (Table 5). TIMP-1 systemic concentration remained constant.

Neither the platelet count nor the prothrombin fragment F1+2 concentration exhibited any changes during the sampling time points. Prior to reperfusion, a modest yet statistically significant increase in the tPA antigen was observed, whereas the values during reperfusion remained stable. The systemic tPA activity variation ranged from 0 to 4 activity units/mL. However, 63% of patient systemic samples exhibited less tPA activity than the method detection threshold. The PAI-1 levels increased significantly before reperfusion and continued to increase during reperfusion, whereas an increase in D-dimer was observed only after commencing reperfusion.

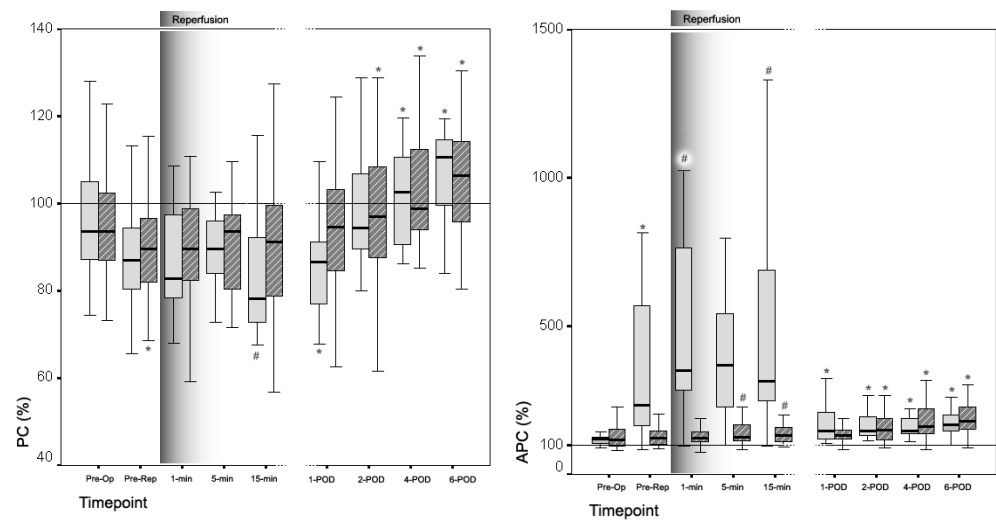
Table 5.

Systemic and transrenal phagocyte activation markers in patients without ATG induction. (* $p < 0.05$ vs. Pre-operative values)

	Pre-operative	Pre-reperfusion	1-min (artery)	5-min (artery)
CD11b (RFU)	242 (127–524)	224 (102–958)*	224 (116–451)	206 (98–650)
L-selectin (RFU)	203 (75–372)	250 (104–394)*	235 (100–412)	230 (109–417)
Lactoferrin (µg/l)	142 (46–260)	130 (55–237)*	118 (41–210)	124 (42–218)
MMP-9 (ng/ml)	23 (6–48)	31 (15–93)*	28 (14–53)	31 (15–193)
TIMP-1 (ng/ml)	157 (90–299)	148 (100–291)	144 (49–273)	151 (87–302)

The systemic blood protein C content exhibited only minor changes during surgery. This was also observed for APC. For both, a more marked increase was observed during the first postoperative week (Figure 7).

Figure 7.
Protein C and activated protein C (APC) during surgery and the first post-operative week.



Adapted from Turunen AJ *et al.* Activated protein C reduces graft neutrophil activation in clinical renal transplantation. *American Journal of Transplantation* 2005; 5: 2204–2212. Reprinted with permission from the copyright holder.

INFLAMMATION IN THE RENAL GRAFT VASCULAR BED DURING REPERFUSION

To simplify the presentation of findings in the following section, we refer to the two blood sampling time points after declamping of the renal graft vessels as “1 min” and “5 min”. The Greek letter Δ represents the transrenal difference in a parameter calculated by subtracting the arterial value from the venous value. Positive values, therefore, indicate graft venous efflux and negative values indicate graft parenchymal or vascular endothelial sequestration. Median values are reported here; the respective ranges of values appear in the original publications in the appendix.

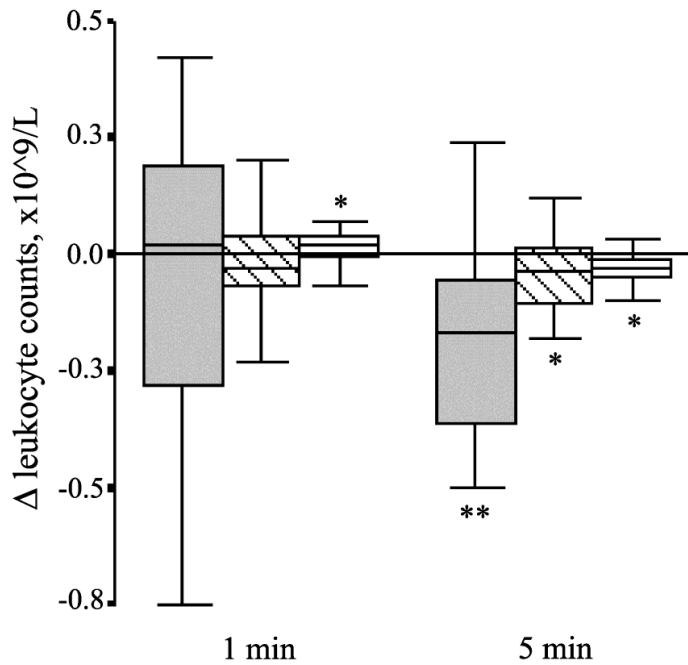
Patients not receiving ATG (groups B and C)

At 1 min, the transrenal cell counts for neutrophils and lymphocytes remained unchanged, while a modest but statistically significant venous efflux of monocytes was observed ($\Delta = 0.02 \times 10^9$ cells/l, $p = 0.04$, Figure 8). Neutrophil CD11b and L-selectin expressions exhibited no transrenal changes and MMP-9 also remained unchanged. Yet, a lactoferrin venous efflux was detected ($\Delta = 15\mu\text{g/l}$, $p = 0.012$). Although transrenal changes to either the neutrophil L-selectin expression or neutrophil count as such were not observed, a significant correlation between the two emerged [$\Delta(\text{L-selectin})$ vs. $\Delta(\text{neutrophil count})$, $R = 0.494$, $p = 0.012$]. In addition, the transrenal MMP-9 difference inversely correlated with a concomitant transrenal neutrophil difference ($R = 0.424$, $p = 0.027$), indicating that a strong renal sequestration of neutrophils was associated with a strong renal release of MMP-9. At 2 min, poor graft blood flow associated with a strong neutrophil sequestration at 1 min ($R = 0.51$, $p = 0.007$).

At 5 min, a marked neutrophil sequestration ($\Delta = -0.17 \times 10^9$ cells/l, $p < 0.001$) in the renal graft was observed (Figure 8). In comparison, only a modest yet still statistically significant sequestration of the lymphocytes ($\Delta = -0.04 \times 10^9$ cells/l, $p = 0.009$) and monocytes ($\Delta = -0.03 \times 10^9$ cells/l, $p = 0.001$) was observed. Neutrophil CD11b and L-selectin expressions remained without transrenal changes. Furthermore, no significant transrenal changes were detected for lactoferrin or MMP-9. The graft blood flow at 30 min correlated with neutrophil sequestration at 5 min ($R = 0.53$, $p = 0.005$, Figure 9).

Figure 8.

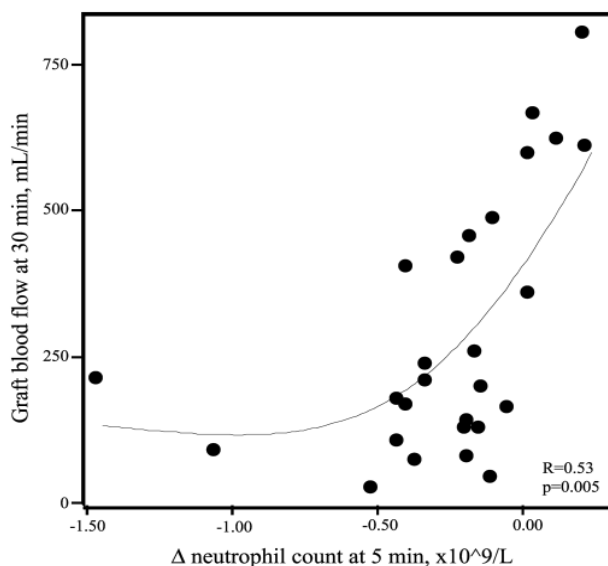
Reperfusion-induced transrenal difference (Δ) of neutrophil (*gray bars*), lymphocyte (*hatched bars*) and monocyte (*white bars*) counts at 1 and 5 min after reperfusion, $\Delta < 0$ implying cell sequestration in the kidney graft. * $p < 0.05$; ** $p < 0.001$.



Adapted from Turunen AJ *et al.* Association of graft neutrophil sequestration with delayed graft function in clinical renal transplantation. *Transplantation* 2004; 12:1821 Reprinted with permission from the copyright holder.

Figure 9.

Graft blood flow correlation to neutrophil sequestration.



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The emergence of DGF was associated with a higher sequestration of lymphocytes, monocytes, and neutrophils at 5 min. To further investigate the relative importance of different leukocyte populations, a binary logistic regression analysis was carried out, where graft neutrophil sequestration emerged as the most important predictor ($p < 0.001$). The association between DGF and MMP-9 appeared moderately significant ($p = 0.05$).

Patients receiving ATG (group A)

Due to the profound systemic leukocyte depletion, the transrenal differences in neutrophil, lymphocyte, or monocyte counts were not evaluated. Similarly, no transrenal differences were found for lactoferrin, MMP-9, or TIMP-1.

COAGULATION IN RENAL GRAFT VASCULAR BED DURING REPERFUSION

Patients not receiving ATG (groups B and C)

At 1 min, significant effluxes from the graft vein were observed for the tPA antigen ($\Delta = 14$ ng/ml, $p < 0.001$) and D-dimer ($\Delta = 58$ ng/ml, $p = 0.001$). A transrenal increase in the tPA activity was also noted ($\Delta = 0.5$ activity units/ml, $p = 0.002$). Yet, the platelet count, PAI-1, F1+2, PC, and APC exhibited no changes.

At 5 min, a significant efflux was detected only for the tPa antigen ($\Delta = 5$ ng/ml, $p < 0.001$), while no transrenal change to the tPa activity was detected. In addition, no significant transrenal changes could be detected for the platelet count, PC, APC, F1+2, or D-dimer. Yet, a significant uptake to PAI-1 was observed ($\Delta = -3$ ng/ml, $p = 0.014$).

Patients receiving ATG (group A)

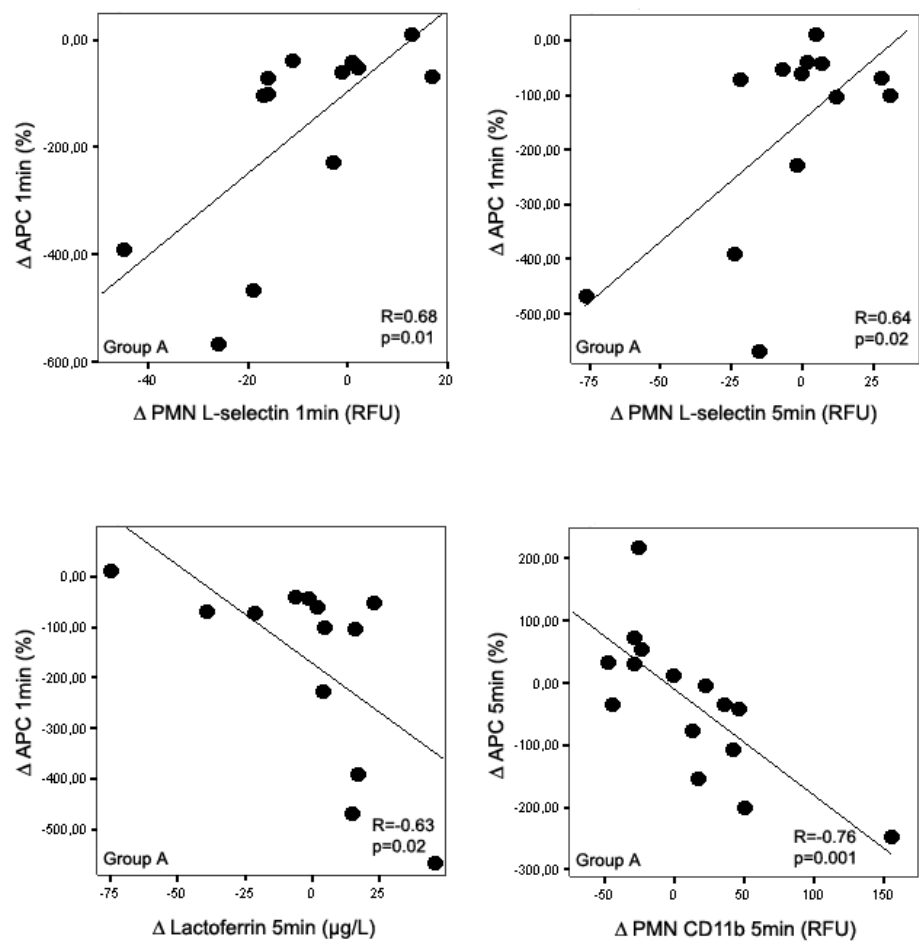
Similar to patients with no ATG exposure, a significant positive venous efflux for tPA antigen at 1 min ($\Delta = 9$ ng/ml) and 5 min ($\Delta = 5$ ng/ml) as well as an efflux for D-dimer at 1 min ($\Delta = 71$ ng/ml) was observed. Contrary to patients with no ATG exposure, a significant PAI-1 venous efflux was observed both at 1 min ($\Delta = 5$ ng/ml) and 5 min ($\Delta = 3$ ng/ml). No transrenal differences, however, were found for the platelet count, F1+2, or the tPa activity.

INTERACTION OF COAGULATION AND INFLAMMATION IN THE RENAL GRAFT VASCULAR BED DURING REPERFUSION

Patients receiving ATG (group A)

The APC transrenal difference at 1 min correlated directly with the neutrophil L-selectin expression transrenal differences at 1 min ($R = 0.7$, $p = 0.01$) and 5 min ($R = 0.6$, $p = 0.02$). Yet, the APC transrenal difference at 1 min correlated inversely with the lactoferrin difference at 5 min ($R = 0.6$, $p = 0.02$). An inverse correlation was also found between the transrenal APC difference at 5 min and the concomitant neutrophil CD11b expression difference ($R = -0.8$, $p = 0.001$, Figure 10).

Figure 10.
Correlations of the transrenal differences for APC with the transrenal differences for the neutrophil activation markers in group A.



Adapted from Turunen AJ *et al.* Activated protein C reduces graft neutrophil activation in clinical renal transplantation. *Am J Transplant.* 2005; 5:2204.Reprinted with permission from the copyright holder.

DISCUSSION

INFLAMMATION WITHIN THE GRAFT VASCULAR BED (STUDIES I AND IV)

Neutrophil degranulation provides us with a trail of information that can be used to measure the established soluble products of neutrophil activation. In this project, a significant lactoferrin venous efflux was detected early at 1 min after vascular declamping (study I). At 5 min, this finding was mirrored by the significant further sequestration of neutrophils in the renal graft. At this point, we estimate that about 4% of the circulating neutrophils was cleared by the renal graft. The relevance of this cell clearance is highlighted by the fact that the increased neutrophil sequestration correlated with a diminished graft blood flow at 30 min after vascular declamping (study I). While other leukocyte populations were also sequestered at 5 min, an association with the blood flow was only found for neutrophil sequestration. This appears to be in line with a classic experimental finding regarding leukocyte capillary plugging causing a regional no-reflow phenomenon (*Engler et al. 1983*). Importantly, DGF also associated with this neutrophil sequestration (study I).

As another product of neutrophil degranulation, MMP-9 is also indicative of neutrophil activation. Significant MMP-9 transrenal changes were absent across all patient groups. Yet, the immense systemic ATG-related elevation of systemically circulating MMP-9 largely obscured the analysis of the renal graft MMP-9 production among group A patients (study IV). However, within group BC, grafts cold-stored for longer periods displayed a venous release of MMP-9. In addition, a strong renal neutrophil sequestration correlated with a strong renal release of MMP-9, suggesting that this MMP-9 release indeed results from trapped neutrophil sources (study IV). Other possible sites of release exist, however, including, for instance, activated glomerular endothelial cells and tubular cells (*Basile et al. 2004*). In this thesis study, an association between MMP-9 release and the emergence of DGF was found (study IV). Damage due to renal ischemic insult has been associated with MMP-9 in experimental settings. Furthermore, in a rat model, ischemia-related microvascular disturbances could be reversed with tetracycline antibiotic, which inhibit MMPs (*Sutton et al. 2005*).

The molecular sequence of the neutrophil recruitment process has been relatively well outlined previously (*Ley 1996, McEver et al. 1995*). Thus, members from both the selectin and integrin families of adhesion molecules were investigated to further elucidate the mechanisms of the neutrophil-associated pathophysiology. After successfully demonstrating the graft neutrophil sequestration, the corresponding results for the adhesion molecules were somewhat surprising, since no statistically significant arteriovenous differences in the

adhesion molecule expressions were detected (study I). However, several factors might contribute to the lack of differences:

Firstly, as L-selectin sheds from the neutrophil surface upon committing to integrin-mediated adhesion, the sampled population of neutrophils may contain both additional activated (low L-selectin, high CD11b) and fewer activated (high L-selectin, low CD11b) cells. Should this be the case, the subpopulations would cancel each other out since the flow cytometric determination of expression actually represents the median expression of any particular neutrophil cohort (*Repo et al. 1993*).

Secondly, it remains unknown to what degree a “touch-and-go” type of activation is possible when blood traverses the capillary vascular beds. It is plausible that the vast majority of activated cells are immediately recruited during the first pass, whereby very little of this population remains available for sampling on the venous side. In this study, L-selectin at 1 min correlated with neutrophil sequestration, providing some indirect support for this reasoning (study I).

Thirdly, numerous means of alternative cell trapping may occur, more or less irrespective of the cell activation status. Experimentally, a low shear stress acts as an autonomous trigger of cell adhesion (*Ploppa et al. 2012*). In addition, the same experiment highlighted various roles for the leukocyte subpopulations in adhesion, since the monocyte presence led to a significant increase in neutrophil adhesion without a notable neutrophil activation. Furthermore, ischemic capillary endothelial cell swelling or sloughing might disturb the flow and affect shear stress, thus increasing neutrophil adhesion.

All of the abovementioned phenomena serve to confound the assays focused on adhesion molecules. In hindsight, measuring the venous efflux of soluble L-selectin during reperfusion would have strengthened the research protocol, an additional consideration that was subsequently examined. After publishing study I, a subsequent analysis among 19 patients (5 in group A, 14 in group BC) examined whether soluble L-selectin is elevated in the graft venous blood. This appeared to indeed be the case among 14 patients not receiving ATG. At 1 min, a statistically significant efflux was present, while at 5 min a similar trend emerged, although it did not reach statistical significance (data not shown).

COAGULATION WITHIN THE GRAFT VASCULAR BED (STUDY III)

Being closely tied to the inflammatory response, unwanted blood coagulation activity threatens graft viability. The heightened incidence of DGF and early graft loss during the first six months post-transplantation was established for an antiphospholipid syndrome–associated prothrombotic state (*Morales et al. 2017*). Many other potential risk factors for thrombosis have been recognized, although established screening or preventive protocols remain lacking (*Andrassy et al. 2004, Parajuli et al. 2016*).

Here, both groups A and BC exhibited a significant venous release of the tPA antigen from the graft during early reperfusion (study III). However, during this time, tPA activity in the graft artery and vein remained static. It is likely that tPA activity is held in check by PAI-1, which is simultaneously sequestered in the graft among group A patients (study III). As a result, the net effect inside the renal vasculature results from antifibrinolysis during early reperfusion. A particularly intriguing finding stems from the tPA-antigen release being complemented by the efflux of D-dimer rather than the efflux of prothrombin fragment F1+2. The absence of F1+2 release indicates that no thrombin formation takes place inside the renal vasculature during cold storage or reperfusion. Together, these markers are, thus, indicative of preformed fibrin lysis rather than the ongoing deposition and lysis of fibrin during early reperfusion (study III).

When, then, did the fibrin deposition and subsequent fibrinolysis take place? The D-dimer venous efflux is significant at 1 min, but not at 5 min. This finding is consistent with washout type kinetics and, as such, might point to the period of cold storage. It is plausible that renal microthrombi form during the intensive care period for the donor. If flushing the graft with preservation solution is unable to clear these clots, fibrinolysis might slowly propagate during the many hours of cold storage. A D-dimer washout is then observed once blood flow is re-established.

Similar behavior among coagulation markers was observed in the systemic samples of brain-dead organ donors (*Lisman et al. 2011*). To our knowledge, our findings here, however, provide one of the most direct pieces of evidence regarding intravascular coagulation and the fibrinolysis of reperfused renal grafts in humans. In the present study, we did not control for pre-existing prothrombotic states, although an independent role for the venous efflux of coagulation markers emerged through a multivariate analysis of DGF risk factors (study III). Contradictory results concerning DGF and microthrombi appear in the literature. In a series of pretransplant donor biopsies, no association was found between donor glomerular microthrombi and DGF (*Sood et al. 2015*). However, *Sood et al.* argued that in their series even grafts with the most widespread glomerular microthrombi represented relatively mild

involvement. Thus, in the histological sections, only 8% of the glomeruli demonstrated more than 50% capillary loop occlusion.

ATG EFFECTS AND SUBSEQUENT APC RENAL UPTAKE (STUDY II)

Most important, this study was designed to study phenomena occurring in the local vascular bed of the renal graft. Thus, the vigorous systemic effects of ATG were largely detrimental. However, an unexpected research opportunity presented itself as novel data concerning the systemic and local effects of ATG could be collected. Given the measured transrenal parameters, patients receiving ATG differed from other patients most notably in relation to APC sequestration. At 1 min after vascular declamping, systemic circulating APC levels had tripled compared with presurgical values. Thus, with a heightened systemic availability, the avid sequestration of APC was observed, whereby 20% uptake by the graft occurred. Importantly, this sequestration correlated with the transrenal markers of neutrophil activation such that an increasing neutrophil activation associated with greater APC uptake. Consumption by the graft endothelium activated or injured via ischemic preservation may offer one explanation, which agrees with previous experimental and clinical results concerning APC (*Lattenist et al. 2016, Petäjä et al. 2001*). The cellular mechanisms possibly behind APC sequestration include ischemia-induced increased thrombomodulin expression on the endothelial cells (*Faust et al. 2001*). Many other modulatory pathways for the renal protective effects of APC have also been suggested, such as cytokine-signaling blockade, cell adhesion control, leukocyte migration, and cell apoptosis (*Gupta et al. 2009*). More recent research found that APC may also stabilize endothelial barriers preventing vascular leakage (*Griffin et al. 2015*). Thus, we may also postulate that the previously observed avid sequestration of APC partially results from capillary leakage into the parenchyma. This leakage then subsequently diminishes when the endothelial barrier functionality is restored by APC, so that at 5 min after declamping it is no longer detected. Regardless of the underlying mechanism, our findings may well represent the most concrete *in vivo* evidence for the anti-inflammatory efficacy of APC in humans.

DELAYED GRAFT FUNCTION (DGF)

At the heart of DGF etiology lies the complex pathophysiology of renal I/R injury (*Siedlecki et al. 2011, Jang & Rabb 2009*). In rodent models, some protective effects have been observed among interventions targeting various leukocyte adhesion molecules (*Fuller et al. 2001*). In humans, an early study utilizing a blocking antibody against intercellular adhesion molecule 1 (ICAM-1) protected against renal ischemic injury (*Haug et al. 1993*), although a randomized prospective study failed to show its efficacy in DGF (*Salmela et al. 1999*). In general, only

limited clinical success largely outside the field of transplantation has been observed (Yonekawa & Harlan 2005).

In this thesis, we found unique evidence for rapid inflammatory activation within the graft associated with subsequent DGF (study I). The magnitude of graft neutrophil sequestration was fourfold that of lymphocytes, in turn indicative of the active mechanisms behind neutrophil sequestration (studies I and IV). From a comparison of pre- and postreperfusion biopsies, histological evidence has linked glomerular neutrophil infiltration to the development of DGF (Koo *et al.* 1998).

In addition, it is unsurprising that the coagulation parameter findings also associated with the resulting DGF. Here, we demonstrated that a high efflux of D-dimer and the efflux of tPA-antigens associated with emerging DGF (study III). Some data do exist concerning the possible direct pro-inflammatory effects of fibrin degradation products (Jennewein *et al.* 2011), but clinical intervention trials in particular presented contradictory results (Jennewein *et al.* 2011). Thus, it is more tempting to interpret the aforementioned associations to be more of implicit quality. Hence, tPA antigen efflux serves as an index parameter for the endothelial injury severity and D-dimer as an index parameter for microthrombi dissolution. While endothelial injury results from brain damage injury as well as cold preservation, our D-dimer results importantly suggest that graft fibrin deposition occurs before organ procurement.

STUDY DESIGN

One of the main challenges to this study stems from the sample size. Once committing to the study, the basic setup of performing a “daughter study” using patients from a larger randomized study seemed advantageous. Thus, we circumvented some of the bureaucracy relating to setting up a clinical study and shared personnel already appointed for the larger study. We estimated that 45 patients would be sufficient to test the underlying hypotheses regarding neutrophil activation and to demonstrate the corresponding adhesion molecule and other laboratory marker changes. This estimate was based only on expert opinion without performing formal power calculations. Early on, it became clear, however, that group A patients’ immunosuppression resulted in early systemic inflammatory behavior quite different to the other two groups and consistent with cytokine release syndrome (Kyllönen *et al.* 2007). While this proved interesting in its own right, this dissimilarity raised immediate concerns regarding the statistical power of detecting more subtle yet important inflammatory marker changes. These concerns proved valid, since we had to combine groups B and C for many analyses in order to meaningfully interpret the results. In many ways, these two groups are similar, although the fundamental disparity in immunosuppression remains. However, we feel

that the likely consequences then would introduce additional confounding factors, thus diminishing the statistical power rather than creating false-positive results.

However, while problematic as described, the ATG-induced systemic inflammatory reaction also allowed for an unexpected research opportunity through an investigation of the extraordinary concentrations of APC in conventionally cold-stored kidney graft vascular beds. This opportunity was fruitfully exploited to produce novel data.

Another study design challenge relates to the differential coefficient nature of the measurements made from the blood samples aspirated locally from the graft vessels. Evaluating the transrenal changes this way, yields freeze-frame information only of a highly dynamic underlying process. Tapping to graft vessels using a cannulation system for several minutes during reperfusion would have provided more integrated results, but was not technically possible at the time. Thus, the integrated magnitude of a particular biomarker sequestration or its release remains open to speculation.

By contrast, compared to obtaining pre- and postreperfusion biopsies, local arterial and venous blood samples likely represent a considerable step forward in our ability to detect rapid changes. In our study, we found that already during the first minutes of reperfusion considerable fluctuations occur in many marker values. We would not expect that a biopsy-based protocol would reveal the early changes in the APC, tPA, or D-dimer values reported here. However, as discussed above, they can be logically explained and, taken with the data on neutrophil sequestration, represent the most insightful pieces of novel information found through this study.

FUTURE PROSPECTS

Clinically, the most noteworthy findings from this study lie in the associations with emerging DGF. As the transplantation community worldwide turns towards ever more compromised organ sources, countering DGF and its consequences remain crucial objectives (*Summers et al. 2015, Heilman et al. 2015*). If donors following cardiac death are utilized, the risk of the graft recipient experiencing DGF doubles (*Summers et al. 2015*). ECD kidneys display a similar inferiority, albeit to a lesser degree. At our institution, the proportion of UNOS extended criteria donors rose from an initial 2.9% during the 1980s to reach 26.3% by 2007 reflecting a global trend (*Salmela & Kyllönen 2007*).

Based on the current literature and the findings presented here, we feel somewhat strongly that the tremendous redundancy in the innate immune activation pathways and effector

functions are likely to prohibit any decisive pharmacological breakthrough in the treatment of renal I/R injury in the near future. Most likely, the most expedient means of improving the prognosis for deceased donor kidneys lies in concerted national efforts aimed at improving deceased donor intensive care. Regrettably, such efforts will not help when non-heart-beating donors are considered. It is highly likely that in the near future these new donor pools will also require consideration in Finland. Thus, further research in this challenging field is very much warranted. With these new donor pools, at the very least, the tedious task of further curtailing the cold preservation time may be possible (*Peeters & Vanholder 2008*). In addition, reconditioning the injured kidney graft with normothermic *ex vivo* perfusion has emerged as an exciting prospect. This approach eloquently bypasses the abovementioned problem of inflammatory redundancy. Instead, a “closed environment” allows the injured graft to regain metabolic activity supported by red cell-based oxygenated perfusate containing no inflammatory cells or soluble mediators (*Hosgood et al. 2017*).

The most appealing future pharmacological intervention might be therapy relying on APC or some other agent with anticoagulation properties. The previous clinical use of APC to treat sepsis was also established as pharmacologically safe in solid organ transplantation setting (*Funk et al. 2011*), thus paving the way for expanding use. Graft vasculature flush treatment, donor pretreatment, or preservation solution supplementation, all represent treatment modalities worth consideration.

In addition, better predicting grafts suffering from DGF would carry immediate clinical value since protective measures could be undertaken to aid jeopardized grafts. Promising predictive techniques have been described, but require refinement for clinical use (*Hattori et al. 2005*, *Schmitz et al. 2008*). Readily available interventions for selected recipients could include the avoidance of nephrotoxic immunosuppressants and timely arrangements for supportive hemodialysis.

Finally, national attitudes towards living donation should be examined, and healthcare practices and policies revised accordingly. Norway undertook a quite different path from Finland, whereby living donation has become common. As minimally invasive surgical donation techniques evolve, short-term ailments for donors considerably diminish. Yet, thorough donor precounseling is mandatory, since minor but real long-term risks remain (*Steiner 2016*). For recipients, the long-term graft prognosis for this type of donation is excellent.

CONCLUSIONS

- I. Our results indicate that neutrophil activation during human renal graft reperfusion is a very rapid phenomenon. Significantly elevated lactoferrin concentration is detected in graft venous blood immediately after vascular declamping. Graft artery and venous blood sampling repeated after five minutes reveals significant ongoing neutrophil sequestration in the graft. This sequestration is associated with diminishing graft arterial blood flow. The patients later diagnosed with DGF have significantly higher renal graft neutrophil sequestration.
- II. Among its' other effects, high dose peroperative ATG administration causes very marked rise of circulating APC. During reperfusion, avid renal graft uptake of APC is observed. APC uptake is associated with renal neutrophil activation and may reflect APC protective role as an endogenous anti-inflammatory mediator.
- III. Results on blood coagulation markers suggest that renal grafts from deceased donors have intravascular fibrin deposits that continue to be degraded during cold preservation. Marked d-dimer venous release during reperfusion is associated with ensuing DGF. These findings imply that future research efforts may be directed towards donor care interventions that take place before organ procurement.
- IV. Surgery itself does not affect MMP9 circulating levels, whereas ATG administration results in very robust systemic rise. While no graft sequestration or release of MMP9 occurs uniformly, the grafts that do display MMP9 wash-out are cold stored for longer periods and are predisposed to develop DGF. A larger focused study is needed to evaluate, whether MMP9 has a unique pathophysiological role or is mere an implicit marker of inflammatory injury.

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My family is my unconditional priority;
Riikka, who walks the earth with me
Silja, Sakari and Olavi, who will take it from there

Hattula, December 2017

The material pressure is just a digressor
- Zack de la Rocha

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